REMARKS

Claims 1, 2, 4, 5, 7-16, 18, 19, and 21-28 were pending in the application. Claims 18, 20, and 21 have been amended. Thus, upon entry of this Amendment, claims 1, 2, 4, 5, 7-16, 18, 19, and 21-28 are pending in the application. Claims 18, 20, and 21 have been amended to change claim dependency.

No new matter has been added. Applicants request that the amendments to the specification and claims be entered. Amendment of the claims should in no way be construed as an acquiescence to any of the Examiner's rejections and was done solely to more particularly point out and distinctly claim Applicants' invention in order to expedite the prosecution of the application. Applicants reserve the right to pursue the claims as originally filed in this or a separate application(s).

Failure of Information Disclosure Statement to Comply With 37 CFR and 1.97(c) and 1.98(a)(2)

The Examiner states that the information disclosure statement (IDS) filed on Dec. 13, 1999 fails to comply with 37 CFR 1.97(c) because it lacks a statement as specified in 37 CFR 1.97(e). The Examiner also states that this IDS fails to comply with 37 CFR 1.98(a)(2) which requires a legible copy of the references submitted.

The publications referred to in the response filed on Dec. 13, 1999 were submitted previously in the IDS filed on August 23, 1999, and were not intended to be submitted as a supplemental IDS. PTO Forms 1449 submitted in the response filed on Dec. 13, 1999 were copies of IDS forms filed on August 23, 1999. Applicants respectfully request that the Examiner withdraw this rejection in view of the IDS that was filed previously in this application.

Claim Objections

Claims 1, 2, 4, 5, 7-11, 13-16, 18, 19, and 21-28 and claim 12 have been rejected for encompassing a non-elected invention. Claims 1, 2, 4, 5, 7-11, 13-16, 18, 19, and 21-28 are directed to a non-human transgenic organism. Claim 12 depends from claim 1 and specifies an organism which is a plant. Claim 12 is a species of genus claim 1, and

therefore is encompassed by the scope of the claimed invention. In view of the Continued Prosecution Application that was filed on July 15, 2002, Applicants submit that the currently pending claims are in order for examination. Applicants respectfully request that the Examiner's rejection regarding the claims in the parent application be withdrawn.

Rejection of Claims 1-11, 13-26, and 27-28 Under 35 U.S.C. §112, first paragraph

Claims 1-11, 13-26, and 27-28 have been rejected under 35 U.S.C. §112, first paragraph. The Examiner states that the specification is enabling for a transgenic mouse, but that it fails to provide enablement for other types of transgenic animals. The Examiner states that

> ...the issue is: following the directions of the specification and the prior art, could an artisan have been able to produce instantly claimed transgenic non-human animals that expressed claimed transgene and produced the same phenotype or what phenotype in the animals and the [the] answer is: due to the unpredictability of the steps and factors of making a transgenic non-human animal, such as, gestation time, litter size, number of fertilized eggs....an artisan would have had to carry out extensive experimentation to make the claimed transgenic non-human animals and such experimentation would have been undue.

The Examiner further states that the Hammer *et al.* reference shows, "...that rat and mice did not show the same phenotype using the same transgene and this is the unpredictability issue that same transgene does not produce same phenotype in two different species." Applicants respectfully traverse this rejection.

Applicants' invention is directed to a non-human transgenic organism having a transgene comprising a polynucleotide sequence encoding a fusion protein which inhibits transcription in eukaryotic cells, the fusion protein comprising a first polypeptide which is a Tet repressor or mutated Tet repressor that binds to a *tet* operator sequence, operatively linked to a heterologous second polypeptide which inhibits transcription in eukaryotic cells. Applicants' invention also encompasses a non-human transgenic organism having a transgene comprising a polynucleotide sequence encoding a fusion protein which inhibits transcription in eukaryotic cells, the fusion protein comprising a first polypeptide which is a Tet repressor or a mutated Tet repressor that binds to a *tet*

operator sequence, operatively linked to a heterologous second polypeptide which inhibits transcription in eukaryotic cells, wherein the transgene is integrated by at a predetermined location within a chromosome within cells of the organism.

The claimed invention also includes a transgenic organism having a transgene integrated into the genome of the organism and also having a *tet* operator-linked gene in the genome of the organism, wherein the transgene comprises a transcriptional regulatory element functional in cells of the organism operatively linked to a polynucleotide sequence encoding a fusion protein which inhibits transcription of said *tet* operator linked gene, the fusion protein comprises a first polypeptide that is a Tet repressor (or a mutated Tet repressor) operably linked to a heterologous second polypeptide which inhibits transcription of said *tet* operator-linked gene in eucaryotic cells, the *tet* operator-linked gene confers a detectable and functional phenotype on the organism when expressed in cells of the organism, the transgene is expressed in cells of the organism at a level sufficient to produce amounts of said fusion protein that are sufficient to inhibit transcription of the *tet* operator-linked gene, and in the absence (or presence) of tetracycline or a tetracycline analogue in the organism, the fusion protein binds to the *tet* operator-linked gene and inhibits transcription of the *tet* operator linked gene, wherein the level of expression of the *tet* operator-linked gene can be upregulated by administering tetracycline or a tetracycline analogue to the organism.

Evidence that the specification is enabled for transgenic organisms other than transgenic mice possessing the Tet-based transcriptional regulatory system system, is provided by references recounting the practice of the described invention by those skilled in the art, including Bello *et al.* (1998) *Dev.* 125:2193-2202; Bieschke, *et al.* (1998) *Mol Gen Genet* 258:571-579; Melfi *et al.* (2000) *J. Mol. Biol.* 304(5):753-763; Ridgway, *et al.* (2000) *Exp. Cell Res.* 256(2):392-399; Weinmann, P. (1994) *Plant J.* 5:559-569 (attached as Appendices A, B, C, D, and E respectively). Applicants point out that Weinmann *et al.* shares authorship with some of the inventors in the instant patent application. These references use substantially the same methods as those described in the instant application, and thus demonstrate that the specification enables one of ordinary skill in the art to practice the claimed invention.

The above-mentioned references describe different transgenic organisms (or organisms containing transgenes in the case of Melfi *et al.* and Ridgway *et al.*), including *Drosophila*, *Xenopus*, sea urchins, and plants, which contain both a first transgene encoding the tetracycline-controllable transcriptional activator and a second transgene containing a *tet* operator-linked gene. In all of the references, excluding Bieschke *et al.*, the tTA used is a fusion protein of the Tet repressor (amino acids 1-207) fused in frame to amino acids 363-490 of the VP16 activation domain. Bieschke *et al.* uses rtTA, where the fusion protein consists of the mutant Tet repressor fused in frame to the VP16 activation domain. These fusion proteins are identical to the tTA and rtTA (or tTA^R) molecules described throughout the specification. Both the tTA and rtTA coding sequences were excised from plasmid pUHD15-1 and pUHD172-1, respectively (described in Examples 1 and 2 at page 54, lines 11-26 and page 55, lines 29-33 of the specification), and inserted into the appropriate vector for transgenic organism construction, using standard methods, vectors, and other reagents known in the art. The *tet* operator-linked gene is controlled by seven tandem *tet* operator sequences upstream of a TATA box. In all of the references (with the exception of Melfi *et al.*) the tet-operator gene construct was created using the seven *tet* operators excised from the plasmid pUHC13-3 (described in Example 2, lines 6-7) and inserted into the appropriate vector for transgenic organism construction, using standard methods, vectors, and other reagents known in the art. Melfi *et al.* uses related plasmid pUHD10-3, which also contains a heptamerized tet operator. Given that the claimed Tet-based transcriptional regulatory system can function in plants, mice, *Xenopus, Drosophila,* and sea urchins, all of which are phylogenetically diverse organisms, it should be expected, and in fact is proven by the above-mentioned references, that the claimed Tet-based transcriptional regulatory system can function in other organisms as well.

The references describe transgenic organisms which contain a transgene for a Tet repressor fused to a transcriptional activator, and are representative of transgenic organisms that contain a Tet repressor fused to a transcriptional inhibitor. The techniques which are used to generate transgenic organisms described in these references can also be applied to creating transgenic organisms containing a Tet-based transcriptional regulatory

system which is used to inhibit transcription of a *tet* operator-linked gene, as those claimed in the instant invention.

Applicants submit that Hammer *et al.* do not teach or suggest that the phenotypic difference between rats and mice containing a B27 transgene is due to differential expression or activity of the B27 transgene in these animals. Applicants' claimed invention is directed to a Tet-based transcriptional regulatory system and is not dependent upon a phenotype that might result from the use of the claimed system. Furthermore, the references described above clearly demonstrate that the claimed invention can be used successfully in transgenic organisms other than mice.

In order to meet the enablement requirement, it is not necessary that a patent specification include specific examples of every different embodiment encompassed by the claims. Moreover, the fact that some experimentation may be necessary to produce transgenic organisms which contain a Tet-based transcriptional regulatory system, does not constitute a lack of enablement as long as the amount of experimentation is not unduly extensive. *Amgen Inc. v. Chugai Pharmaceutical Co., Ltd.*, 927 F.2d 1200, 1213 (CAFC 1991). A considerable amount of experimentation is permissible if it is merely routine, or if the specification provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *In re Wands* 8 USPQ2d 1400-1407, 1404 (CAFC, 1988). In the instant case, in view of the guidance provided by the specification, Applicants have enabled claims 1-11, 13-26, and 27-28. Accordingly, Applicants respectfully request that the rejection of claims 1-11, 13-26, and 27-28 under 35 U.S.C. §112, first paragraph, be withdrawn.

Rejection of Claims 1-11 and 13-28 Under the Judicially Created Doctrine of Obviousness-Type Double-Patenting

Claims 1-11 and 13-28 have been rejected under the judicially created doctrine of obviousness-type double patenting over U.S. Patent No. 5,866,755. Applicants will submit a terminal disclaimer upon agreement and acceptance of the claims in the instant application by the Examiner.

CONCLUSION

In view of the foregoing remarks, reconsideration of the rejections and allowance of all pending claims is respectfully requested.

If a telephone conversation with Applicants' attorney would expedite the prosecution of the above-identified application, the examiner is urged to call Applicants' attorney at (617) 227-7400.

Respectfully submitted,

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

18. **(Amended)** The organism of claim 16 ~~17~~, wherein the first polypeptide comprises an amino acid sequence shown in SEQ ID NO: 17.

19. **(Amended)** The organism of claim 16 ~~17~~, wherein the first polypeptide of the fusion protein is a mutated Tet repressor that binds to *tet* operator sequences in the presence but not the absence of tetracycline or a tetracycline analogue.

21. **(Amended)** The organism of claim 19 ~~20~~, wherein the mutated Tet repressor has at least one amino acid substitution compared to a wild-type Tet repressor.

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Spatial and temporal targeting of gene expression in *Drosophila* by means of a tetracycline-dependent transactivator system

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SUMMARY

In order to evaluate the efficiency of the tetracycline-regulated gene expression system in *Drosophila*, we have generated transgenic lines expressing a tetracycline-controlled transactivator protein (*tTA*), with specific expression patterns during embryonic and larval development. These lines were used to direct expression of a *tTA*-responsive promoter fused to the coding region of either the β-galactosidase or the homeotic protein Antennapedia (ANTP), under various conditions of tetracycline treatment. We found that expression of β-galactosidase can be efficiently inhibited in embryos and larvae with tetracycline provided in the food, and that a simple removal of the larvae from tetracycline exposure results in the induction of the enzyme in a time- and concentration-dependent manner. Similar treatments can be used to prevent the lethality associated with the ectopic expression of ANTP in embryos and, subsequently, to control the timing of expression of the homeoprotein ANTP specifically in the antennal imaginal disc.

Our results show that the expression of a gene placed under the control of a tetracycline-responsive promoter can be tightly controlled, both spatially by the regulatory sequences driving the expression of *tTA* and temporally by tetracycline. This provides the basis of a versatile binary system for controlling gene expression in *Drosophila*, with an additional level of regulation as compared to the general method using the yeast transcription factor GAL4.

Key words: Tetracycline, Gene expression, *Drosophila*, *Antennapedia*

INTRODUCTION

An essential and general experimental approach to analyse the function of a gene in a whole organism is to examine the phenotypic consequences of its directed expression in certain cells, or at a developmental stage, in which the gene is normally silent. In *Drosophila*, two major systems have been designed to achieve the conditional expression of gene constructs integrated into the genome. In the first, the coding sequence of the gene of interest is placed under the control of promoters inducible according to the culture conditions. The *hsp70* gene promoter is commonly use for that purpose (see Schneuwly et al., 1987 and references therein). High levels of induction can be obtained at well-defined time periods during development upon exposure of the organism to elevated temperatures. This advantage is often limited because ectopic expression occurs in all cells and endogenous genes are repressed during heat shocks. Consequently, side defects including lethality may mask the result of the ectopic expression in the desired cell type(s). It is also frequently necessary to repeat heat shocks over an extended time period to observe the phenotypic consequences, and it may be difficult to distinguish between the primary and secondary effects of the overexpression (e.g. Gibson and Gehring, 1988).

An alternative approach is to engineer a gene construct inducible by a single transcription factor whose activity can be controlled *in vivo*. The ability of the yeast transcription factor GAL4 to activate transcription in *Drosophila* (Fisher et al., 1988) has been exploited to generate a versatile method for targeting gene expression in this organism (Brand and Perrimon, 1993). Any gene of interest can be placed under the control of a promoter containing GAL4 upstream activating sequences (UAS) and integrated stably into the genome of a parental line (responder strain), since it remains silent in the absence of GAL4. Tissue-specific expression of the gene is obtained upon crossing to a second parental line (driver strain) expressing the transcription factor under the control of a suitable promoter. Although a large number of strains expressing GAL4 in a wide variety of patterns can be selected on the basis of the expression of a reporter gene bearing the UAS sequences (Brand and Perrimon, 1993; Yeh et al., 1995; Calleja et al., 1996), only a few of them have been used to direct expression of functional proteins in the post-embryonic stages of development (Brand and Perrimon, 1993; Capdevila and Guerrero, 1994; Hinz et al., 1994; Speicher et al., 1994; Rimmington et al., 1994; Ferver et al., 1995; Halder et al., 1995; Zink and Paro, 1995; Freeman, 1996; Morimura et al., 1996). The limit in this binary system lies in the lack of

temporal control, which remains primarily determined by the regulatory sequences driving GAL4. Because most of the gene-specific enhancers are active at various stages of development in *Drosophila*, GAL4-mediated induction is frequently observed from the embryonic stage onwards, and often results in premature lethality (e.g. Halder et al., 1995). To overcome this difficulty, Flp-mediated recombination has been used to achieve conditional expression of a transgene upon recombination of an FRT cassette separating the coding region from a promoter (Struhl and Basler, 1993). This approach can be used to control the expression of GAL4 (Pignoni and Zipursky, 1997). In any case it requires the combination of various specific constructs and it can be applied only to tissues in which cell division occurs, since it results in the generation of clones of expressing cells.

Extension of the more versatile binary system for post-embryonic studies could be achieved with the use of a regulatory protein modulated in the organism with an innocuous effector. One of the best candidates is the tetracycline-dependent transactivator (*tTA*) comprising the tetracycline repressor of *E. coli* (*tetR*) and the strong transcriptional activation domain of the herpes simplex virus protein VP16 (Gossen and Bujard, 1992). The high affinity and specific binding of *tetR* to the tetracycline operator sequences (*tetO*) can be inhibited by tetracycline (Hillen and Wissmann, 1989) and is thought to result from a conformational change of *tetR* upon association with tetracycline (Hinrichs et al., 1994). In HeLa cells, *tTA* was found to stimulate transcription of a promoter bearing a multimerized *tetO* by several orders of magnitude, and a fast and reversible switch of the *tTA*-dependent promoter was obtained upon addition or removal of tetracycline from the culture medium (Gossen and Bujard, 1992). These features have been extensively exploited in tissue culture where tetracycline levels can be tightly controlled. Tetracycline-regulated expression of reporter genes was also demonstrated in whole plants (Weinmann et al., 1994) and in transgenic mice (Hennighausen et al., 1995; Kistner et al., 1996). In the latter case, the efficiency of tetracycline, administrated by slow-release tetracycline pellets or in drinking water, has been mostly evaluated by analysing the expression of sensitive reporter genes, although a few examples of successful expression of proteins have been reported (see Shockett and Schatz, 1996 for a review).

In this study we determined the functional properties of *tTA* in *Drosophila* by expressing this regulatory protein under the control of various promoters. Using a *lacZ* reporter gene placed under the control of a promoter bearing seven copies of *tetO*, we found that expression of β-galactosidase can be tightly controlled in embryos and larval tissues. Furthermore, we used *tTA*-expressing strains and tetracycline treatments to drive tissue-specific ectopic expression of the homeoprotein *Antennapedia (Antp)* at different stages of development. Our results demonstrate the usefulness of the *tet* system in *Drosophila*.

MATERIALS AND METHODS

DNA constructs

Most of the constructs were assembled by multiple step cloning according to standard methods (Sambrook et al., 1989). Further details and maps are available upon request.

tTA driver constructs

hsp70-tTA

The *tTA* coding region was isolated as a 1.1 kb *Eco*RI-*Bam*HI fragment from pUHD 15-1 (Gossen and Bujard, 1992) and cloned into CaSpeR-hs (Thummel and Pirrotta, 1992)

RHT (*rosy*, *hsp70* promoter, *tTA*)

This P element vector derives from the enhancer-test vector HZ50PL (Hiromi et al., 1985). The *tTA* coding region is flanked by the minimal promoter and the poly(A) sequences of *hsp70* (Fig. 1A).

ey-tTA and *HoxA7-tTA*

The *eyeless (ey)* gene enhancer (a 3.5 kb *Kpn*I fragment from *ey* Eco 3.6) (B. Hanck, T. Eggert, W. J. Gehring and U. Walldorf, unpublished) and a 630 bp fragment of the intron of the *HoxA7* gene from pB6 (Haerry and Gehring, 1996) were cloned in RHT to give *ey-tTA* and *HoxA7-tTA*, respectively.

Tetracycline-responder constructs

tetO-lacZ

The heptameric repeat of the tet operator was isolated as a 310 bp *Eco*RI-*Kpn*I fragment from pUHC 13-3 (Gossen and Bujard, 1992) and cloned upstream of the P-*lacZ* fusion of the enhancer-test vector CPLZ (Wharton and Crews, 1993). CPLZ contains the P-element transposase promoter (up to –42 from the cap site) and the N-terminal transposase sequence fused in-frame with *lacZ* and the polyadenylation signal of *hsp70*.

WTP (white-*tetO*-P promoter)

This P-element vector was constructed to express any gene under the control of a tetracycline-responsive promoter. It contains the vector backbone of CPLZ, the heptameric repeat of the tet operator, the P-element promoter and leader sequences from Carnegie 4 (Rubin and Spradling, 1983) and the polyadenylation signal of SV40.

tetO-Antp and *tetO-AntpΔHD*

The cDNAs encoding a full-length ANTP protein or a variant with a deletion of the homeodomain were isolated as *Not*I fragments from pHSSΔA and pNHT-A11, respectively (Gibson et al., 1990) and cloned into the corresponding site of WTP.

Germline transformation and *Drosophila* strains

P-element mediated transformation of ry^{506} or *y ac* w^{1118} recipient strains was carried out essentially as described (Spradling, 1986). A description of the markers and balancer chromosomes indicated in Figs 3 and 4 can be found in Lindsley and Zimm (1996). A405.1 M2 and rK781 have been described (Wagner-Bernholz et al., 1991; Flister, 1991).

Tetracycline media and treatments

A tetracycline-containing medium suitable for larval feeding and maintenance of adults was obtained by mixing 100 ml of tetracycline solution (tetracycline hydrochloride (Sigma) diluted with sterile water at the required concentration), 25 g of Instant *Drosophila* Food (Carolina Biological Supply) and 1 g of dry yeast.

Tetracycline was provided to adult females by placing 50-70 virgins in a glass vial containing a 2.5 cm filter (Whatman grade 3 MM) soaked with 500 μl of a 4% sucrose solution with tetracycline at the appropriate dilution. Females were fed for 3-4 days, following a daily cycle of 16 hours on tetracycline-containing filters and 4 hours on standard food supplemented with a drop of yeast paste. Males of the relevant genotype were placed with females on tetracycline and allowed to mate overnight before the beginning of egg collections. For

experiments requiring larval feeding, batches of 100-200 eggs harvested from grape juice plates were placed on pieces of nylon mesh, and allowed to develop at 25°C on tetracycline-containing food. When necessary, larvae were separated from their food by floating in 30% glycerol, collected with forceps, washed with PBS and transferred on standard food in groups of 50 to 100.

Phenotypic analyses

Embryos and larval tissues were fixed and stained for β-galactosidase as described (Bellen et al., 1989). Adult heads were separated from the body of narcotised flies and holes were made into the cuticle to facilitate penetration of the fixative. For antibody staining of embryos and examination of cuticular phenotypes, standard procedures were applied (Ashburner, 1989).

RESULTS

We have designed a general system to express *tTA* under the control of regulatory sequences (RHT driver construct), in order to direct the expression of a gene of interest under the regulation of a tetracycline-responsive promoter (WTP responder construct). The gene constructs can be stably propagated into the genome of separate strains, and *tTA*-dependent gene induction is obtained in the F_1 offspring of the cross where it can be controlled by tetracycline (Fig. 1A).

tTA is a potent transactivator in *Drosophila*

In order to analyse the transactivation potential of *tTA*, we have used an indirect heat shock assay to drive ubiquitous expression of *Antp* in embryos. Heat shock assays were performed on embryos carrying *tTA* under the control of the *hsp70* gene promoter (*hsp70-tTA*) and a WTP derivative carrying a full-length *Antp* cDNA (*tetO-Antp*). Independent transformants were found to give an identical embryonic phenotype to the H4 line that carries a direct *hsp70-Antp* construct (Fig. 1B; see also Gibson and Gehring, 1988 for a complete description of the H4 line). In contrast, heat-shocked embryos carrying a WTP derivative with a deletion of the homeodomain (*tetO-Antp*ΔHD) or the empty WTP vector (*tetO–*) showed a wild-type cuticle and developed to the adult stage (Fig. 1B and data not shown). A western blot of embryonic extracts prepared from heat-shocked embryos and probed with an ANTP-specific monoclonal antibody (Condie et al., 1991) reveals similar levels of ANTP expression (Fig. 1C). In addition, transformation of the adult antenna into a mesothoracic leg can be obtained when heat shocks are applied to third instar larvae (Gibson and Gehring, 1988; D. Resendez-Perez, B. Bello and W. J. Gehring, unpublished). Taken together, these results show that *tTA* can activate transcription of a promoter that contains *tetO* sequences without any toxic effect of this regulatory protein in *Drosophila*.

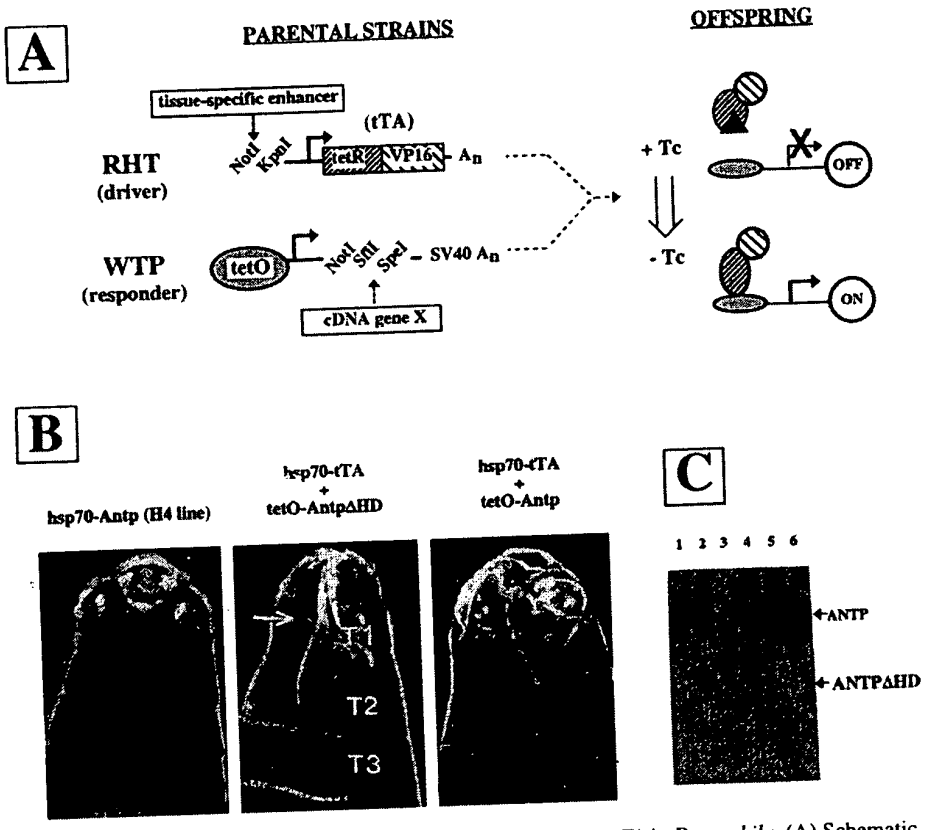

Fig. 1. Transgenic constructs and the transactivation potential of *tTA* in *Drosophila*. (A) Schematic representation of the tetracycline-inducible system in *Drosophila*. The transformation vectors RHT and WTP are, respectively, designed to express *tTA* under the control of enhancer-like elements and any cDNA under the regulation of a *tTA*-responsive promoter that contains *tetO* sequences. Derived constructs are integrated and propagated in two separate parental strains. In absence of tetracycline (–Tc), the binding of *tTA* results in the activation of gene X with the spatial and temporal specificities determined by the driver construct (on state). Raising the offspring on a tetracycline-containing medium is used to prevent the binding of *tTA* in order to keep the responder construct silent up to a certain point of time (off state). Stopping tetracycline exposure allows time-specific induction of gene X upon withdrawal of the antibiotic. (B) Cuticular preparations of embryos heat shocked for 30 minutes at 6.5 hours of embryogenesis. All embryos carry a single copy of the constructs indicated at the top. Note the similar defect in head evolution in *hsp70-Antp* and *hsp70-tTA*; *tetO-Antp*, and the T1 to T2 transformation when compared to the wild-type cuticle of *hsp70-tTA*; *tetO-Antp*ΔHD embryos. The identical segmental transformation is revealed by the disappearance of the characteristic denticle belt of the first thoracic segment (white arrow), indicating a transformation of T1 towards T2. The head segments are also transformed towards T2. T1, T2, T3: first, second and third thoracic segments respectively. (C) Detection of the ANTP proteins by western blot analysis in crude extracts of embryos heat shocked for 2 hours with a 4 hour recovery. Lane 1, *hsp70-Antp* embryos (H4 line); lanes 2- 6, embryos carrying *hsp70-tTA* and either *tetO-Antp* ΔHD (lane 2) or the empty WTP vector (lane 3), or independent insertions of *tetO-Antp* (lanes 4-6).

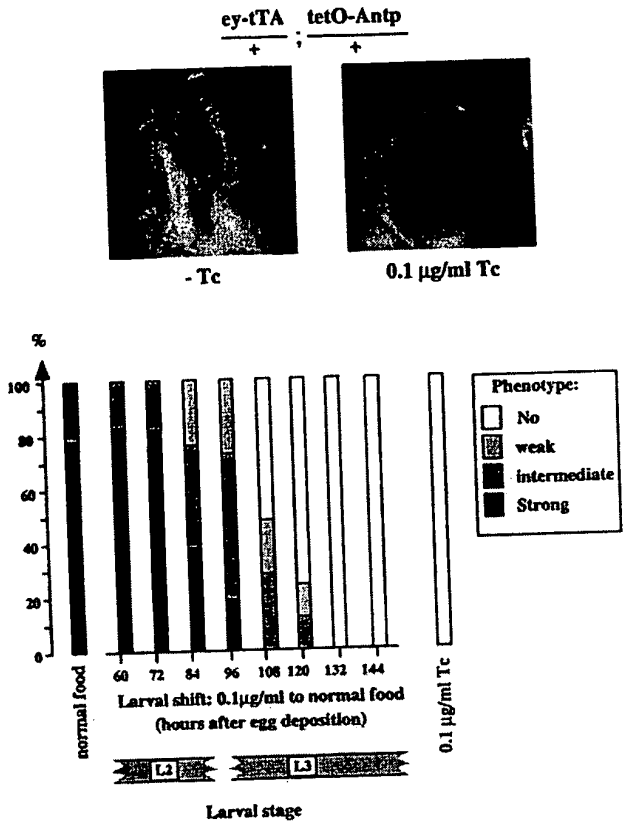

Fig. 3. Induction of *Antennapedia* in the eye imaginal disc by means of tetracycline. (Top) Eyes of adult flies raised in absence (–Tc) or in the presence of 0.1 µg/ml tetracycline (Tc) in the larval food. (Bottom) Semi-quantification of the eye phenotype in adults derived from larvae raised with 0.1 µg/ml tetracycline and shifted to standard food at 12 hour intervals. At least 50 adults were scored at every time point. Larval stages were identified on the basis of the morphology of the tracheal system and the anterior spiracles.

a comparable staining intensity in imaginal discs isolated from larvae exposed to tetracycline and control larvae. These data show that the withdrawal of the larvae from tetracycline is followed by a rapid induction of the *tetO-lacZ* transgene under the control of *tTA*. The concentration- and time-dependent expression of *lacZ* is likely to reflect the need to lower the concentration of tetracycline in the disc cells below a certain threshold level, which allows *tTA* to bind the tet operator and to stimulate transcription of the promoter. A careful examination of the staining patterns also suggested that the *tetO-lacZ* transgene was not turned on in every cell at the same time after withdrawal of tetracycline (Fig. 2A). To confirm this observation, we performed larval shifts during the second half of the third instar, when expression of *tTA* is uniform in the posterior part of the eye disc. Upon removal of the larvae from 0.1 µg/ml tetracycline, induction of *lacZ* can clearly be observed in a gradually increasing number of cells (Fig. 2B). Similar observations were obtained with different *tTA*-expressing strains, suggesting variations in the kinetics of the clearance of the antibiotic and/or the trancriptional activation.

Tetracycline-controlled expression of *Antennapedia* targeted to the eye disc

To determine the relevance of the data obtained with the *lacZ* reporter gene we used the same driver strain to direct expression of *Antp* under various conditions of tetracycline treatment. In the absence of tetracycline, adults obtained from

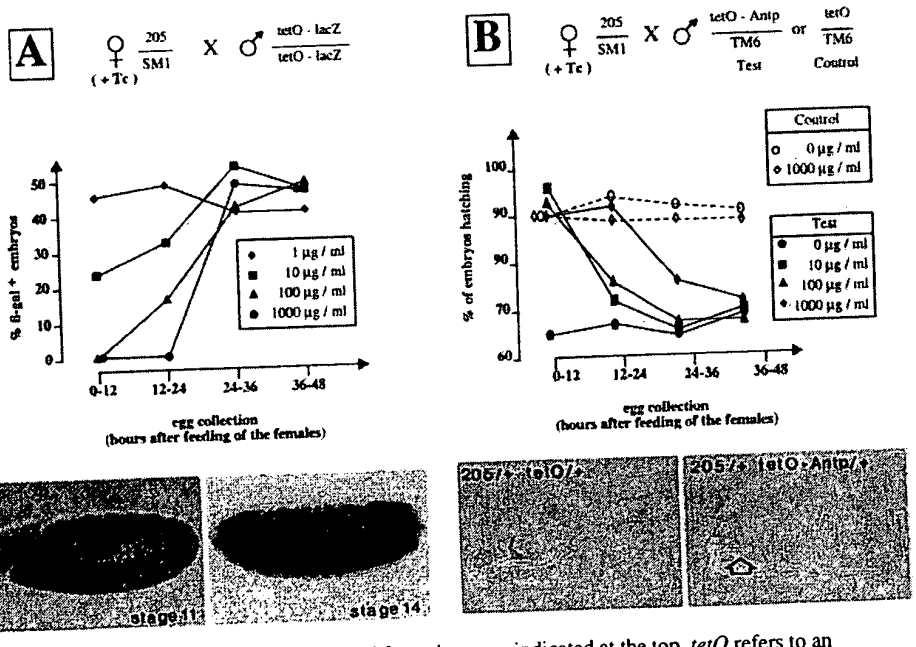

Fig. 4. Inhibition of *tTA*-dependent gene expression in embryos by maternal transmission of tetracycline. (A) Repression of the *lacZ* reporter driven by the 205 strain, shown at the bottom by immunodetection of β-galactosidase. Embryos collected over successive 12 hour periods following the feeding of 205/SM1 females with tetracycline, were stained for β-gal activity. At least 100 embryos older than the germ band extended stage (stage 11) were scored at every time point. 50% of transheterozygotes were expected in the offspring of the cross indicated at the top. (B) Tetracycline-dependent rescue of embryonic lethality by repression of the *tetO-Antp* transgene. Embryos collected over 12 hour periods after feeding of their mothers with tetracycline were allowed to develop at 25°C and the percentage of hatching larvae determined. At least 200 embryos were scored at every time point. 25% of transheterozygotes are expected from the cross indicated at the top. *tetO* refers to an insertion of the empty vector WTP on the third chromosome, used as a control. Bottom: cuticular phenotype of embryos of the indicated genotype. Note the failure of head involution marked by the open arrow in embryos expressing *Antp* under the control of the 205 strain.

a cross between the parental *ey-tTA* and *tetO-Antp* strains showed reduced and irregular compound eyes (Fig. 3, –Tc). Since *Antp* is normally not expressed in the eye disc (Wirz et al., 1986), its expression in this tissue would interfere by unknown mechanisms with the normal development of the eye. No other morphological defects could be detected in adults, in agreement with the eye-specific expression of *tTA* detected with the *lacZ* reporter. To ascertain that the eye phenotype resulted from the ectopic expression of *Antp* in the eye disc, we raised larvae on a medium containing tetracycline at various concentrations. The eyes were restored to a wild-type appearance with 0.1 μg/ml tetracycline (Fig. 3, top) but not with 0.01 μg/ml tetracycline, in good agreement with the dose-dependent repression of the *lacZ* reporter (Fig. 2A).

As indicated with the *lacZ* reporter, the directed expression of *Antp* by the *ey-tTA* strain should occur continuously throughout larval development and shift rapidly during the third instar when the cells undergo differentiation. We used tetracycline to control the timing of *Antp* overexpression in order to determine the functional significance of this dynamic pattern of expression, with respect to the alteration of the eye development. Newly hatched larvae were first fed with 0.1 μg/ml to inhibit *tTA* activity and then transferred to a standard medium every 12 hours to induce *Antp*. Adults were scored for eye defects and classified according to an arbitrary scale of strong, intermediate, weak or no detectable eye phenotype (Fig. 3, bottom). All the adults derived from larvae exposed to tetracycline up to the second instar showed strong eye defects in a range indistinguishable from their siblings raised in absence of tetracycline. In contrast, subsequent shifts allowing induction of *Antp* from the early third instar onward led rapidly to a complete rescue of the eye morphology. These results confirm the efficiency of the tetracycline control with a functional homeoprotein and suggest that the alteration in the normal eye development is mostly dependent on the ectopic expression of *Antp* in the undifferentiated cells of the eye epithelium.

Repression of *tTA*-dependent gene expression in embryos by maternal transmission of tetracycline

The embryonic development of *Drosophila* is not easily amenable to antibiotic treatment since the egg is protected by an impermeable set of eggshells but it is relatively fast (22-24 hours at 25°C) and maternal components are transmitted to the oocyte by the nurse cells and the follicle cells in the female ovaries. The influence of tetracycline given to the parental females was first tested on the strong *lacZ* expression driven by the *tTA* construct of the line 205 (Fig. 4A). This driver line 205 was isolated among twelve independent transformants of the *HoxA7-tTA* construct (see Materials and Methods) because of its unique expression pattern observed in the antennal disc (Fig. 5), the leg discs, the central nervous system, the epidermis and various internal tissues (not shown). Since the other lines showed a reproducible pattern in the eye disc and the larval brain due to the *HoxA7* enhancer (not shown), the line 205 is likely to reflect a modified expression of the transgene under the influence of genomic regulatory sequences flanking the integration site (Wilson et al., 1990). When assayed in embryos with the *lacZ* reporter gene, expression of *tTA* in the 205 strain starts at the end of germ band extension, about 5 hours after egg laying (AEL), and is detected mostly in the trunk region with a segmentally repeated pattern (Fig. 4A, bottom). This pattern changes rapidly, so that at the end of germ band retraction (approximately 10 hours AEL), strong expression is detected all over the ectoderm. The staining appears patchy in the cephalic segments and is not uniformly distributed in the thoracic and abdominal segments (Fig. 4A, bottom). After treatment of the females with tetracycline (see Materials and Methods), repression of the *lacZ* reporter is mostly effective in the eggs collected immediately after the end of exposure to the antibiotic and is dose-dependent (Fig. 4A, top). Repression was obtained in 100% of the eggs collected within 12 hours after the treatment of the females with 100 μg/ml or more of the antibiotic, and in a large fraction of them with 10 μg/ml. The gradual loss of repression observed in the eggs collected later is likely to reflect a decrease of the maternal pool of tetracycline accompanying the continuous production of eggs.

The same procedure of tetracycline treatments was tested for its effect on the survival rate of embryos carrying the driver construct 205 and either a *tetO-Antp*, or an empty responder construct (*tetO–*), as control (Fig. 4B). Examination of the embryonic cuticles revealed major defects in the formation of the head (Fig. 4B, bottom), a phenotype reminiscent of heat-shocked embryos in which *Antp* was ubiquitously expressed (Fig. 1B), although no homeotic segmental transformations were observed. This might reflect a different level of induction of *Antp* as compared to the use of a heat shock promoter but it is more likely to be due to a difference in the timing and the spatial expression of the homeoprotein, since transformations obtained by heat shocks are optimal when induced at 5-7 hours of development (Fig. 1B, see also Gibson and Gehring, 1988), at a stage when the 205 driver is mostly active in the trunk and is not ubiquitously expressed (Fig. 4A, bottom). Nevertheless, line 205 allowed us to test the effect of tetracycline on the survival rate of the embryos and, as shown in Fig. 4B, the embryonic lethality could be overcome in a dose-dependent manner by providing tetracycline to the females. The embryonic rescue was in good agreement with the tetracycline-mediated repression of the *tetO-lacZ* transgene (Fig. 4). These two independent assays clearly demonstrate the possibility to inactivate *tTA* in embryos in order to keep a promoter silent during this stage of development.

Targeted mis-expression of *Antp* in larvae following embryonic rescue

The efficient inactivation of *tTA* in embryos prompted us to analyse the fate of tetracycline-rescued embryos in more detail. As expected, embryos did not develop to the adult stage in the absence of tetracycline in the larval food, whereas addition of tetracycline led to the recovery of viable adults in a concentration-dependent manner (the quantitative data are available upon request). In addition, larvae raised under optimal conditions were shifted to standard food at various times to allow induction of *Antp*. Shifts performed during the late third instar led to the recovery of pharates or adults, whereas larvae shifted earlier essentially failed to undergo metamorphosis. Examination of the adults revealed very specific morphological modifications of the antennae and the head vertex (Fig. 5K-M), in the area expressing the *lacZ* reporter under the regulation of the 205 driver line (Fig. 5E-G). In contrast, transheterozygotes raised continuously with 10 μg/ml tetracycline showed wild-type structures (Fig. 5H-J),

demonstrating the highly specific alterations in the development of adult flies following the mis-directed expression of *Antp* by the 205 line. As revealed with the *lacZ* reporter, expression of *tTA* follows a dynamic spatial pattern in the primordia of the antenna from the mid-third instar onwards (Fig. 5A-D) and in the presumptive area of the occelli from the larval/pupal transition onward (Fig. 5C,D). Moreover, the lag in induction imposed by the removal of the larvae from tetracycline exposure suggests that the alterations of adult structures mostly result from the ectopic expression of *Antp* during the pupal stage. No defects were found in the legs or the palps where the 205 driver is also strongly expressed (not shown), in agreement with previous observations showing that only the derivatives of the eye-antennal disc respond to the ubiquitous expression of *Antp* induced by heat shock (Gibson and Gehring, 1988). These latter studies showed that repeated pulses of heat-shock expression are required during the third larval instar to achieve complete antenna to leg transformations. Our results confirm previous observations showing that the late larval induction of *Antp* does not induce fully differentiated morphological markers of the leg (Scanga et al., 1995; Larsen et al., 1996). Although we observe different arrangements of bristles on the antenna, none of them showed the bracts characteristic for leg bristles.

Directed expression of *Antp* in the antennal disc by *tTA* activates *rK781*

Since the observation of adult phenotype required late larval shifts, we asked whether the consequences of *tTA*-dependent expression of *Antp* could be directly assayed in the imaginal discs. As a marker, we used the enhancer detector line rK781, which was isolated in a screen for *Antp*-regulated genes on the basis of their response to the overexpression of the ANTP homeoprotein in the eye-antennal disc (Wagner-Bernholz et al., 1991). We combined driver, responder and test constructs in larvae, exposed them to tetracycline treatment, and assayed β-galactosidase expression in wandering third instar larvae. When raised continuously with 10 μg/ml tetracycline, the normal pattern of rK781 expression was detected in all the discs (not shown) and in a few cells of the antennal disc (Fig. 6, left), as previously described (Wagner-Bernholz et al., 1991; Flister, 1991). When dissected from larvae that were removed from tetracycline exposure, *lacZ* expression could be reproducibly detected in the form of a crescent at the border between the arista and the third antennal segment (Fig. 6, middle). This area corresponds to the most proximal part of the wedge-shaped sector expressing *tTA*, as visualised with the *lacZ* reporter (Fig. 6, right) and is also the first to express *tTA* during third instar (Fig. 5B). These results show that derepression of *Antp* by removal of tetracycline can be demonstrated by the activation of a downstream target gene in the antennal disc. These findings indicate that the tetracycline-dependent expression system efficiently repressed *Antp* in embryos and allows subsequent derepression in imaginal discs.

DISCUSSION

In this study, we report a detailed evaluation of the different properties of the tetracycline-dependent gene expression system in *Drosophila*. Since its description in transformed HeLa cells (Gossen and Bujard, 1992) this regulatory system has been extensively used in cell culture. In higher eucaryotes including plants and mouse, tetracycline-controlled activity of *tTA* has been mostly evaluated on reporter genes (see Shockett and Schatz, 1996 for a review), although a few examples of successful expression of proteins have been reported (Efrat et al., 1995; Ewald et al., 1996; Mayford et al., 1996; Shockett et al., 1995; St-Onge et al., 1996). By using *Drosophila* lines expressing high levels of *tTA*, we show that the strong induction of the *lacZ* reporter can be efficiently prevented by tetracycline in both embryos and imaginal discs. We have evaluated the dose-response and defined easy and reliable protocols of tetracycline treatment to control the repression of the *lacZ* reporter gene. Furthermore, we also show that this system is fully functional to control the spatial and temporal expression of the ANTP homeoprotein. The lines *ey-tTA* and 205 described in this study show the highest levels of *tTA* among the lines generated to date in the laboratory and we have reproducibly obtained repression of gene activity in embryos by feeding their mothers with tetracycline in a range from 1 to 1000 μg/ml tetracycline, and in larvae, with as little as 0.1 μg/ml tetracycline. The use of tetracycline is especially appropriate to keep the inducible gene promoter silent during embryogenesis in order to direct its expression during larval development. Tetracycline concentrations ranging from 0.1 to 10 μg/ml ensure reactivation of the tetracycline-responsive promoter within 24 hours after transfer of the larvae to normal medium. It is important to point out that the amount of tetracycline required to inactivate *tTA* is both low and non-toxic. This is essential to keep the promoter inactive up to a desired stage and to ensure its fast activation upon removal from tetracycline exposure. We have found that the addition of tetracycline to the larval food does not give any toxic effect in a range of 0 to 100 μg/ml, although the development is slowed down at concentrations above 1 μg/ml. As shown with the *ey-tTA* strain, tetracycline can be used to control the timing of induction at distinct phases of development in order to define a phenocritical period. Temporal control of gene expression should also be effective during pupal development as a function of the concentration of tetracycline provided to the larvae before pupation. They can be well synchronised during this developmental period and go through a number of well-characterised stages (Ashburner, 1989). In combination with the use of the *lacZ* reporter, these features should help in determining the time course of induction of any gene driven by a *tTA*-expressing line of interest.

Our attempts to use the reverse tetracycline-controlled transactivator (*rtTA*, Gossen et al., 1995) have been unsuccessful in *Drosophila*. This transactivator is based on a mutagenized version of *tTA*, which binds the *tetO* sequences only in the presence of specific tetracycline derivatives. It corresponds to a 4-amino-acid exchange in *tetR*, which is thought to alter the conformation of the repressor and allows its binding to DNA upon association with certain tetracycline compounds (Gossen et al., 1995). Since it was originally isolated in a genetic screen in bacteria and tested successfully in mammalian cells (Gossen et al., 1995) and in transgenic mice (Kistner et al., 1996), *rtTA* might need a temperature close to 37°C to be stable. In contrast, *tTA* shows a potent activity in *Drosophila* and its negative regulation by tetracycline is not a major difficulty, as described above. Furthermore, both the repression and the kinetics of gene induction might be

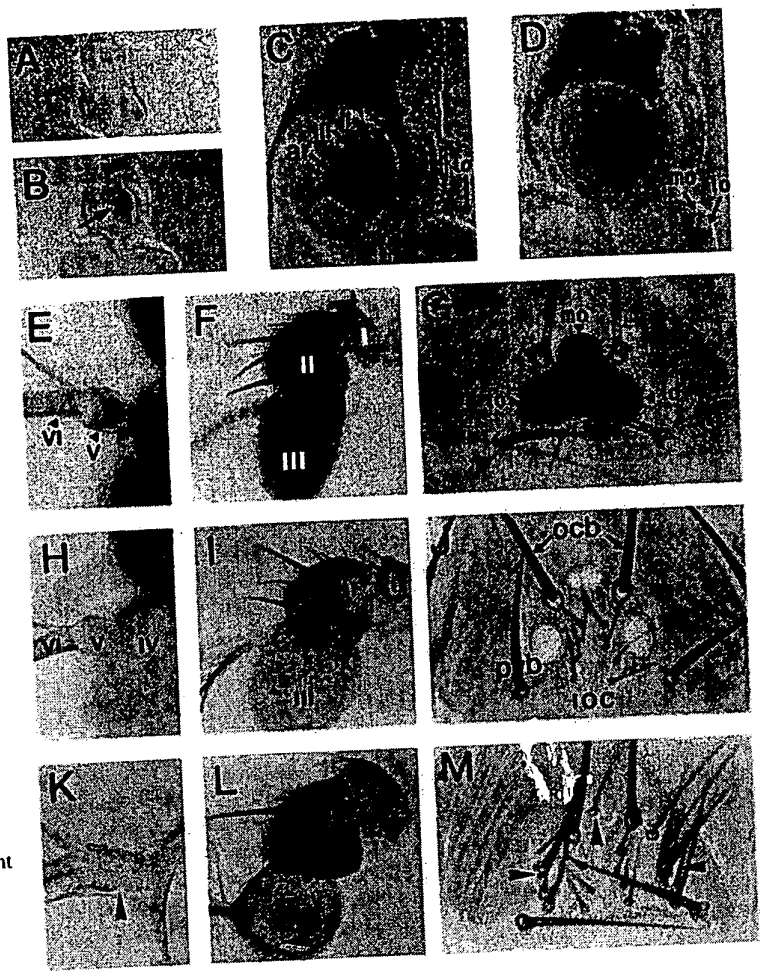

Fig. 5. Adult phenotype resulting from the directed expression of *Antennapedia* by the 205 strain. (A-D) Expression pattern of the 205 strain in the eye-antennal disc visualized by X-gal detection of β-galactosidase activity encoded by *tetO-lacZ* reporter. Discs are oriented with anterior up and dorsal left. (A) Early third instar. (B) Mid third instar; expression starts in the centralmost region of the antennal disc (arrow) and the presumptive palp region (arrowhead). (C) Late third instar larvae: expression has expanded in a wedge-shaped sector overlapping the most proximal region of the arista (ar) and the three major antennal segments (roman numerals). Expression in the lateral occellus (lo) that is not detectable in active wandering larvae is also indicated. (D) White prepupae: β-gal activity is detectable in the medium occellus (mo). (E-G) Expression of the *lacZ* reporter in the respective adult structures: the most proximal segments of the arista (E), the three antennal segments (F) and the occelli (G). (H-J) Normal phenotype of 205/+; *tetO-Antp*/+ adults raised continuously with 10 μg/ml tetracycline. Occipital, post vertical and interoccellar bristles are indicated by ocb, pvb and ioc, respectively. (K-M) Altered phenotype of 205/+, *tetO-Antp*/+ adults derived from larvae shifted from 10 μg/ml tetracycline to standard food during the late third instar. Note the thickening of the proximal segments of the arista (K, arrowhead), a bunch of new bristles on the third antennal segment and a modification in the number, the localization and the shape of the characteristic bristles of the second antennal segment (L, arrowheads). We could not determine the origin of these bristles on the basis of their morphology. Although different from the usual antennal bristles, they are not of a leg type because of the lack of the characteristic bract. The other main feature is a bunch of thick and long bristles of unknown origin close to the occelli (M, arrowheads).

increased further with one of the numerous tetracycline derivatives available. Some of them have been shown to be more potent effectors on *tTA* than tetracycline itself (Gossen and Bujard, 1993; Chrast-Balz and Hooft van Huijsduijnen, 1996).

Binary systems for controlling gene expression

The interest in using a binary system that combines an effector molecule for controlling activity of a responder promoter has been largely demonstrated with the GAL4/UAS system (Brand and Perrimon, 1993). Our method makes use of a similar experimental strategy, which allows the stable integration of any kind of construct in a parental fly strain in the absence of the transactivator. Tissue-specific activation of the gene construct is achieved in the offspring of a cross with a driver line chosen for its pattern of expression of the transactivator. The *tet* system provides a more versatile tool, owing to the possibility of controlling the timing of gene expression during development. In addition, the use of the vectors RHT and WTP facilitates the generation of driver strains expressing *tTA* under the control of previously isolated tissue-specific enhancers, and responder strains carrying any gene of interest under the regulation of the *tetO*-containing promoter. We also plan to generate a collection of *tTA*-expressing strains following the

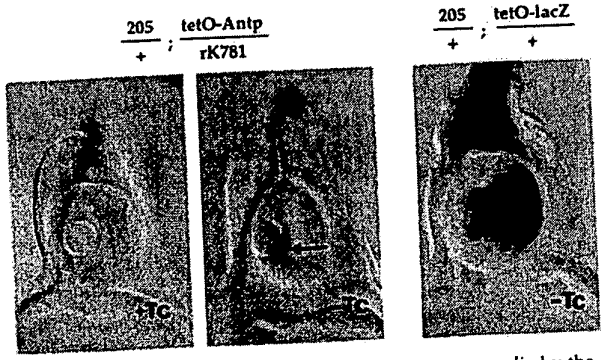

Fig. 6. Tetracycline-controlled expression of *Antennapedia* by the 205 strain activates rK781. Shown are β-galactosidase-stained antennal discs isolated from larvae raised continuously on 10 μg/ml tetracycline (+Tc) or shifted to standard food 48-72 hours before dissection (−Tc). The arrow points to the activation of rK781 in the centralmost region of the wedge-type sector of *tTA* expression, visualized on the right by the *tetO-lacZ* reporter. Eggs were collected over 12 hours from tetracycline-treated females of the genotype Cy[A405. M2]/205: rK781/rK781 mated with +/+ ; *tetO-Antp*/TM6,Tb males. Larvae of the genotype 205/+ ; *tetO-Antp*/rK781 were identified by their Tb^+ phenotype and the absence of the staining pattern due to the Cy[A405. M2] chromosome.

random integration into the genome of an enhancer detector construct with *tTA* as a reporter gene. The availability of strains expressing *tTA* in a wide variety of patterns will ensure a large number of applications for the *tet* system.

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ORIGINAL PAPER

Appendix B

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Doxycycline-induced transgene expression during Drosophila development and aging

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Abstract The "reverse" tetracycline repressor (rtR) binds a specific DNA element, the tetracycline operator (tetO), only in the presence of tetracycline, or derivatives such as doxycycline (dox). Fusion of rtR to the transcriptional activation domain of herpes virus protein VP16 produces a eukaryotic transactivator protein (rtTA). rtTA has previously been shown to allow dox-dependent transcription of transgenes linked to tetO sequences in mammals. To adapt this system to *Drosophila*, the *Actin5C* promoter was used to drive constitutive expression of rtTA in transgenic flies. Three reporter constructs, each encoding *E. coli* β-galactosidase (β-gal), were also introduced into transgenic flies. In one reporter seven tetO sequences were fused to the *Adh* core promoter. The other two reporter constructs contain seven tetO sequences fused to the *hsp70* core promoter. Feeding of transgenic *Drosophila* containing the rtTA construct and any one of the three reporter constructs with dox caused up to 100-fold induction of β-gal. Dox induced β-gal expression in all tissues, in larvae and in young and senescent adults. Induction of β-gal in adults had no detectable effect on life span. These results suggest the potential usefulness of this system for testing specific genes for effects on *Drosophila* development and aging.

Key words Tetracycline repressor · Inducible promoter · Drosophila · Aging

Introduction

Inducible gene expression systems have long been an important tool in analyzing the function of specific genes in bacteria, yeasts, and *Drosophila*. In *Drosophila*, inducible transgenic systems usually rely wholly or in part on the use of a heat shock protein (hsp) gene promoter, which is transcriptionally induced in response to heat stress (Lis et al. 1983). While hsp gene promoters have been used to great advantage in many experiments, the system has several important limitations. First, the heat stress required for induction can have pleiotropic effects, including developmental abnormalities (phenocopies) (Lindquist 1986) and reduced fertility and viability. This is a problem particularly in experiments designed to study the aging process, since life span will be dramatically affected by changes in fertility and viability (Tower 1996). Another situation where the use of a heat-inducible promoter is problematic is the analysis of the heat shock proteins themselves. It is not possible to induce expression of a single hsp and study its effects, without the complication of inducing the entire endogenous repertoire of hsps. This problem has been overcome in certain experiments by using the metallothionein promoter (Petersen and Lindquist 1988; Solomon et al. 1991), which is inducible by heavy metal ions. However, the metallothionein promoter functions only in specific gut cells in transgenic *Drosophila* (Otto et al. 1987), thus limiting its potential usefulness. Therefore, there is a need for an alternative inducible gene expression system in *Drosophila*.

In the last five years, efficient inducible gene expression systems have been developed for mammalian systems based on the *E. coli* tetracycline repressor (tetR) (Gossen and Bujard 1992; Furth et al. 1994). The tetR binds to its target sequence, the tetracycline operator (tetO) only in the absence of the antibiotic tetracycline. The first system developed for mammals was the "tet-off" system (Gossen and Bujard 1992; Furth et al. 1994; Shockett et al. 1995). tetR protein was fused with the transcriptional activation domain of herpes virus transcription factor VP16. In the absence of tetracycline this protein binds to tetO sequences placed within the promoter of a gene of interest, thereby driving transcription. Addition of tetracycline then prevents binding and stops transcription ("tet-off"). A "tet-on" system was created

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by generating a mutant tetR:VP16 fusion protein, which had the reverse property of only binding to the tetO and activating transcription in the presence of tetracycline ("tet-on") (Gossen et al. 1995; Kistner et al. 1996). We report here the successful adaptation of the "tet-on" system to transgenic *Drosophila*.

Materials and methods

Plasmid constructions

Plasmid rtTA (reverse-tetracycline Trans Activator) was constructed by first inserting the 850-bp *Hind*III-*Xba*I fragment from pCaSpeR-AUG/β-gal (Thummel et al. 1988), containing the SV40 splice and poly(A) signals, into the *Hin*dIII (partial restriction digestion) and *Xba*I sites of the polylinker of the pCaSpeR4 transformation vector (Thummel and Pirotta 1992), to generate plasmid cSV. Plasmid pUHD172–1neo (Gossen et al. 1995) was digested with *Eco*RI, endfilled with T4 polymerase, then digested with *Bam*HI, to liberate a 1-kb fragment containing the reverse-tetracycline trans-activator coding sequence. Plasmid cSV was digested with *Spe*I, endfilled with T4 polymerase, then digested with *Bam*HI, and the 1-kb fragment from pUHD172–1neo was inserted, to generate the plasmid cTSV. DNA sequencing of cTSV revealed that it had resulted from an unexpected ligation event: the *Eco*RI site from the inserted fragment was conserved in this cloning step, and the 1-kb fragment was actually inserted into the *Bam*HI site, without any change in the *Spe*I site. The *Actin5C* promoter was inserted into plasmid cTSV in several steps. First, plasmid D237 (also called "Act5C > Draf+ > nuc-lacZ"; Struhl and Basler 1993) was digested with *Not*I, endfilled with T4 polymerase, then digested with *Kpn*I, and the resultant 4.3-kb fragment containing the *Actin5C* promoter was inserted into the *Kpn*I/*Eco*RV sites of pBlueScript II KS (Stratagene), to generate pAc. The 4.3-kb *Actin5C* promoter fragment was liberated from pAc by restriction digestion with *Kpn*I and *Eco*RI, and inserted into the *Kpn*I/*Eco*RI sites of cTSV, to generate the plasmid cATSV. DNA sequencing revealed that the *Actin5C* promoter in plasmid cATSV was in the wrong orientation relative to the reverse-tetracycline transactivator coding region. To correct this, the *Actin5C* promoter region was liberated by digestion with *Eco*RI, and then re-inserted into the same *Eco*RI site. DNA sequencing was used to identify a construct with the *Actin5C* promoter in the correct orientation, which was then named plasmid rtTA.

The seven tandem repeats of the tetO region in plasmid pUHC13–3 (Gossen et al. 1995) were amplified by PCR using the primers: 5′-TCGACTGCAGCTTTCGTCTTCAAGAATTCCTCGAG-3′ and 5′-AGCTTCTAGATACACGCCTACTCGACCCGGGTACCGAG-3′. The 367-bp PCR product was digested with *Pst*I and *Xba*I at the sites engineered into the primers, and then inserted into the *Pst*I/*Xba*I sites of pBlueScript II, to generate plasmid p7T.

Plasmid 7TAdh was constructed as follows. Plasmid pAdh/β-gal (Irvine et al. 1991; Koelle et al. 1991) was partially digested with *Eco*RI, and then completely digested with *Pst*I to liberate a 4.8-kb fragment containing the *Adh* basal promoter region (positions −33 to +53), the *Ubx* 5′ leader sequences fused to *lacZ*, and the SV40 splice and poly(A) signals. This fragment was cloned into the *Pst*I/*Eco*RI sites of the pCaSpeR4 polylinker, to generate plasmid pCaSpeR-Adh/β-gal. The 359-bp *Pst*I-*Xba*I fragment from plasmid p7T, containing the heptameric tetO region, was then inserted into the *Pst*I/*Xba*I sites of pCaSpeR-Adh/β-gal, to generate plasmid 7TAdh.

Plasmid 7T40 was constructed as follows. Construct c70Z (Simon and Lis 1987) was digested with *Hin*dIII and *Eco*RI to liberate a fragment containing the *hsp70* promoter fused to *E. coli lacZ*. This *Hin*dIII-*Eco*RI fragment was cloned into the *Hin*dIII/*Eco*RI sites of plasmid pBS2N to generate plasmid pBS2N′. Plasmid pBS2N is pBlueScript II KS + (Stratagene) in which the unique *Kpn*I site has been converted to a *Not*I site (a gift of L.R. Bell, University of Southern California). Construct c70Z was also digested with *Eco*RI alone to liberate an *Eco*RI fragment containing the *hsp70* poly(A) signal sequences, and this fragment was cloned into the unique *Eco*RI site of plasmid pBS2N′ to generate plasmid pBS2N″. Plasmid pBS2N″ was digested with *Hin*dIII and *Apa*I, treated with exonuclease III and with nuclease S1, and then ligated. The resultant plasmid was called c40Z, and DNA sequencing revealed a 5′ *hsp70* promoter deletion to position −40 relative to the start site of transcription. Plasmid c40Z is one of a series of *hsp70* 5′ promoter deletions which will be described in detail elsewhere (J. C. Wheeler and J. Tower, unpublished data). Plasmid c40Z was digested with *Not*I to liberate a 3.7-kb fragment containing the entire 5′Δ-40 *hsp70:lacZ* fusion gene, and this fragment was cloned into the *Not*I site of p7T, to generate plasmid p7T40-pre. A fragment containing the seven tetO repeats and the entire 5′Δ-40 *hsp70:lacZ* fusion gene was liberated from p7T40-pre by digestion with *Xho*I and *Spe*I, then inserted into the *Xho*I/*Spe*I sites in the polylinker of pCaSpeR4, to generate plasmid 7T40.

Plasmid 7TAUG was constructed as follows. A 4.6-kb SalI fragment from pCaSpeR-AUG/β-gal (Thummel et al. 1988), containing the *Adh* translation initiation sequence fused to *lacZ* and the SV40 splice and poly(A) signals, was cloned into the *Sal*I site of pBlueScript II KS, to generate plasmid pAUG. A *Pst*I fragment from plasmid 7T40, containing the seven tetO repeats and the *hsp70* promoter from −40 to +86, was inserted into the *Pst*I site of pAUG, to generate plasmid p7TAUG. A fragment containing the seven tetO repeats and the entire *hsp70:lacZ* fusion gene was liberated from p7TAUG by digestion with *Xho*I, and inserted into the *Xho*I site of pCaSpeR4, to generate plasmid 7TAUG.

Drosophila culture

Fly stocks were maintained on cornmeal/agar medium (Ashburner 1989). To obtain adult flies of defined ages, stocks were cultured at 25° C until 0–2 days post-eclosion, and then males only were transferred to 25° C or 29° C as indicated in Figure legends. These males were maintained at < 50 per vial and transferred to fresh vials every 2–4 days. Double transgenic adult males were obtained by crossing males of a transactivator stock (rtTA) to virgins of the reporter stocks (7TAdh, 7T40, and 7TAUG). Transgenic flies were generated by standard methods (Rubin and Spradling 1982), using the w^{1118} recipient strain.

Doxycycline treatments

Young flies (5–7 days post-eclosion) and old flies (28–32 days post-eclosion) were treated with the tetracycline derivative doxycycline hydrochloride (dox) (Sigma) by feeding. The indicated concentration of dox, in 20 mM Tris (pH 7.5) containing 10% sucrose, was soaked into a single Kim-Wipe (Kimberly-Clark), in an empty *Drosophila* culture vial. After feeding with dox for the specified time, the flies were returned to cornmeal/agar food vials, and allowed to recover as indicated. For treatment of larvae, the cornmeal/agar medium was supplemented with dox to a final concentration of 0.25 mg/ml, prior to seeding of the culture.

Spectrophotometric assay of β-galactosidase activity

β-Galactosidase (β-gal) activity was quantitated in whole fly extracts using published procedures (Simon and Lis 1987). Assays were performed under conditions in which the reaction was linear with regard to the amount of extract. Data are presented as the average +/− the standard deviation for triplicate assays. Protein concentration of extracts was determined using the Bradford reagent (BioRad). The w^{1118} strain was used to generate all transgenic lines, and no β-gal activity was detectable in extracts of the w^{1118} strain using the spectrophotometric assay.

In situ staining for β-galactosidase activity

β-galactosidase expression was visualized in dissected flies, larvae, and cryostat sections using published procedures (Simon et al. 1985).

Results

Basic components of the system

To achieve tetracycline-inducible induction of transgenes in all tissues it is necessary that the reverse tetracycline transactivator (rtTA) be expressed in all tissues. The rtTA is a fusion of the reverse tetracycline receptor (rtR), which binds to DNA only in the presence of tetracycline, with the transcriptional activation domain of herpes virus transcription factor VP16. In construct rtTA, the constitutive *Drosophila Actin5C* promoter was used to drive expression of the rtTA coding region. This construct also contains the SV40 poly(A) signal sequence (Fig. 1A). To test the system, three reporter constructs were generated, each encoding *E. coli* β-gal. The constructs differed in the source of the core promoter, 5′ UTR, and polyadenylation signal sequences in order to maximize the chances of generating a construct which could yield high-level transgenic protein expression in *Drosophila*. In the first reporter construct (7TAdh) seven tetO sequences are fused to the *Adh* core promoter, followed by the *Ubx* 5′ untranslated region, the *E. coli lacZ* coding region, and the SV40 poly(A) signal (Fig. 1B). A regulatory element composed of seven tetO sequences was chosen because this element was previously shown to function in transgenic mice (Kistner et al. 1996). In the second reporter (7T40), the seven tetO sequences are fused to the *hsp70* core promoter, and *hsp70* 5′ untranslated region, followed by the *E. coli lacZ* coding sequences and the *hsp70* poly(A) signal (Fig. 1C). In the third construct (7TAUG), the seven tetO sequences are fused to the *hsp70* core promoter, followed by the *Adh* 5′ untranslated region, the *lacZ* coding region, and the SV40 poly(A) signal (Fig. 1D). Multiple independent transgenic lines were generated for each construct. Each line is homozygous for the transgenic construct, and is designated by the name of the construct followed by the chromosome in which the construct is inserted (in parenthesis), followed by a letter/number combination for each independent transgenic line. For example, line 7TAdh(2)A2 is transgenic line number A2 and has the 7TAdh construct inserted on the second chromosome.

Flies were then generated which contained both the rtTA construct and the 7TAdh construct ("double-transgenic" flies). This was done by crossing flies of stock rtTA(2)C1 to flies of stock 7TAdh(2)A2, which yields progeny containing one copy of each construct. A sample of these double transgenic flies were fed sucrose solution containing 1.0 mg/ml dox for 48 h, while the controls were fed sucrose solution alone. The flies were allowed to recover for 3 days, then sectioned using a cryostat, and the sections were stained for β-gal activity (Fig. 2A). In the treated flies robust β-gal activity (blue stain) was detected in all tissues. In the control flies, low-level β-gal activity was detected primarily in the gut, and thus the system allows dox-induced transgene expression in all tissues of the adult. The same results were obtained with transgenic flies containing the other two reporter constructs, 7T40 and 7TAUG (data not shown).

To determine if the system also works during development, line rtTA(2)C1 was crossed again to reporter line 7TAdh(2)A2, and also to reporter line 7T40(3)B1, and the larvae from each cross were cultured on food containing 0.25 mg/ml dox, and on

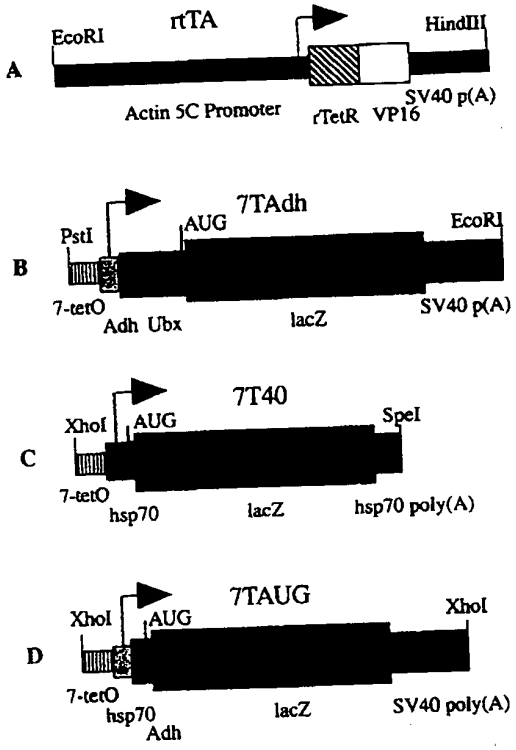

Fig. 1A–D Transgenic constructs. Each construct fragment shown is cloned into the indicated restriction sites of the polylinker of the pCaSpeR-4 transformation vector. The assembly of each construct is described in detail in Materials and methods. Diagrams are not to scale. **A** rtTA. The constitutive *Actin5C* promoter and 5′ untranslated region are fused to the coding sequences for the rtTA (reverse tetracycline transactivator), which is a fusion of the rtR (reverse tetracycline repressor) and the transcriptional activation domain of herpes virus protein VP16. The poly(A) signal sequences are from SV40. **B** 7TAdh. Reporter construct consisting of seven tetO sequences, the *Adh* core promoter, the *Ubx* 5′ untranslated region and translational initiation sequence, the *E. coli lacZ* coding region and the SV40 poly(A) signal sequences. **C** 7T40. Reporter construct consisting of seven tetO sequences, the *hsp70* core promoter, 5′ untranslated region and translational initiation sequence, the *E. coli lacZ* coding region and the *hsp70* poly(A) signal sequence. **D** 7TAUG. Reporter construct consisting of seven tetO sequences, the *hsp70* core promoter, the *Adh* 5′ untranslated region and translational initiation sequence, the *E. coli lacZ* coding region, and the SV40 poly(A) signal sequence

Fig. 2A–C Dox-induced transgene expression detected by an in situ β-gal activity assay. A rtTA transgenic line rtTA(2)C1 was crossed to reporter line 7TAdh(2)A2. Young adult progeny were fed with either control sucrose solution (*upper panel*) or sucrose solution containing 1.0 mg/ml dox (*lower panel*) for 48 h, and then allowed to recover for 3 days. Flies were sectioned on a cryostat, and stained for β-gal activity using the chromogenic substrate X-gal. In the control (*upper panel*), low-level β-gal activity is detected primarily in gut tissues. The gut staining indicates some leakiness of expression in the absence of dox, as in non-transgenic *Drosophila* only very faint gut staining is detectable, and only in the abdomen (data not shown, see also Wheeler et al. 1995). In dox-treated (*lower panel*), β-gal activity is detected in all tissues, with the exception of the central region of the indirect flight muscles. All of the indirect flight muscle tissue stains intensely if the staining reaction is allowed to continue for a longer period (data not shown). However with longer staining times the increased intensity of stain in the other body segments obscures the detail of specific tissues, and therefore the results for the shorter staining time are presented. **B** Progeny from the cross rtTA(2)C1 × 7T40(3)B1 were cultured on standard *Drosophila* culture media (upper larva) or *Drosophila* media containing 0.25 mg/ml dox (lower larva). Whole third-instar larvae were stained in situ for β-gal activity. No β-gal activity was detected in the control tissue larvae (*upper larvae*), or in non-transgenic larvae (data not shown). General β-gal activity was detected in the dox treated larvae (*lower larvae*). **C** Repeat of the experiment in **B**, using progeny of the cross rtTA(2)C1 × 7TAdh(2)A2. β-Gal expression in larvae with this reporter was reproducibly less efficient than in the experiment shown in **B**

control food. As seen in Fig. 2B, C, staining of whole third-instar larvae revealed high-level, tissue general induction of β-gal activity with reporter 7T40(3)B1, and somewhat lower level, tissue general induction with reporter 7TAdh(2)A2. The dox-fed larvae were also observed to be slightly smaller than the controls, which may be due to a toxic effect of the dox and/or β-gal expression during development.

Characterization of the response

The induction of β-gal expression can be quantitated by spectrophotometric assay of β-gal activity in fly extracts. This assay was used to optimize the time course of dox treatment. Transactivator line rtTA(2)C1 was crossed to reporter line 7TAdh(3)D1, and the double transgenic progeny were treated with 1.0 mg/ml of dox for 24 h, 48 h, and 48 h plus varying times of recovery without dox. As seen in Fig. 3A, 48 h of treatment plus 3 days of recovery gave the optimal degree of induction (~10-fold). With greater times of recovery, β-gal activity decreased, indicating that the induction is reversible upon withdrawal of dox. The same result was obtained using a different reporter stock, containing the 7T40

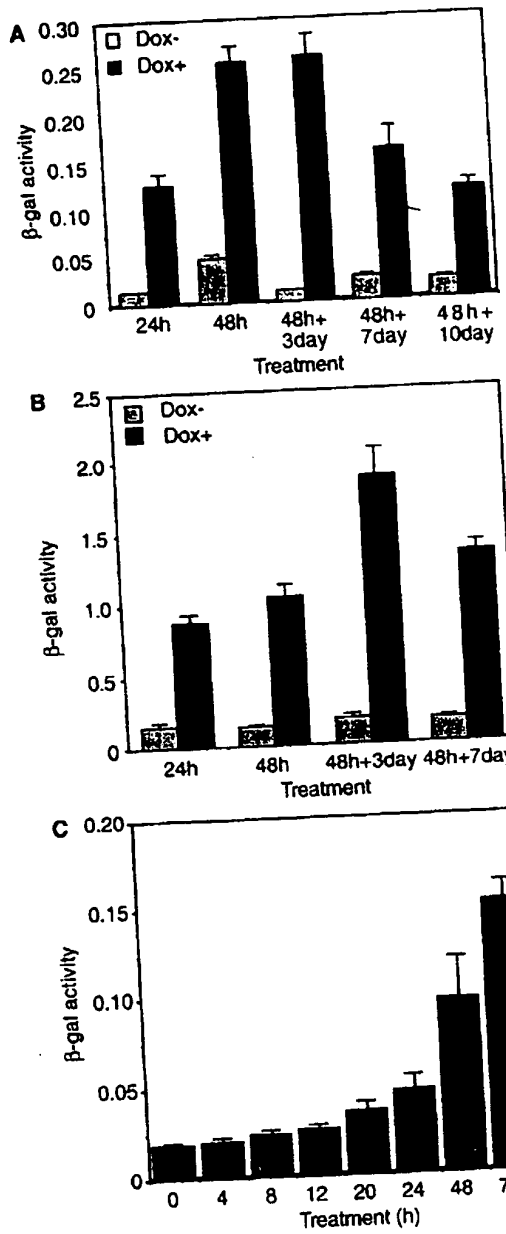

Fig. 3A–C Time course of transgene induction by dox. A Young adult progeny of the cross rtTA(2)C1 × 7TAdh(3)D1 were mock treated (Dox-; *stippled bars*), or treated with 1.0 mg/ml dox (Dox+; *black bars*) for the indicated time periods, and allowed to recover as indicated. Triplicate samples containing three flies each were homogenized, and β-gal activity was quantitated using the spectrophotometric assay. β-Gal activity is expressed in relative units, and the averages +/- SD are presented. B The experiment in A was repeated with a different reporter stock, using young adult progeny from cross rtTA(2)C1 × 7T40(2)E1. C The experiment in A was repeated using progeny from cross rtTA(2)C1 × 7TAdh(2)A2, and the timecourse for induction was analyzed in greater detail

construct, 7T40(2)E1 (Fig. 3B). Thus, both the *hsp70* core promoter and the *Adh* core promoter can respond to activation by the tetO sequences and the rtTA transactivator.

In transgenic mice the activation by the rtTA transactivator can be quite rapid, with activation by several orders of magnitude occurring in the first 4 h, and maximum levels of activation being achieved by 24 h (Kistner et al. 1996). The timecourse of activation in *Drosophila* was analyzed in greater detail (Fig. 3 C), and found to be significantly slower. In the progeny of the cross rtTA(2)C1 × 7TAdh(2)A2, induction of β-gal by dox feeding was quantitated at intervals between 4 and 72 h. Significant activation was not detected until 8–20 h, and maximal induction required ≥72 h. Similar results were obtained with construct 7TAUG (data not shown). Note that while the level of induction and timecourse was similar for the different reporters in Fig. 3, they are not identical. This probably reflects small differences in the activities of the different reporter insertions, as well as the variability inherent in working with live adult *Drosophila* and administration of dox by feeding.

The *Drosophila* tet-on system was next characterized for the dose response to dox (Fig. 4). Double transgenic adults (rtTA(2)C1 × 7TAdh(2)A2) were fed dox for 48 h and allowed to recover for 3 days (Fig. 4, open circles), or for 96 h plus a 3-day recovery period (closed circles). For 48-h treatment times, β-gal activity was found to increase in response to dox concentrations from 0.01 to 2 mg/ml. Use of the longer 96-h treatment time allowed equivalent levels of β-gal expression with one-tenth as much dox. Thus, longer treatment times reduce the amount of dox required for efficient induction.

To compare the relative activities of the three different reporter constructs, two independent transgenic lines for each reporter were crossed to the rtTA(2)C1 transactivator line (Fig. 5A). Dox-induced β-gal expression

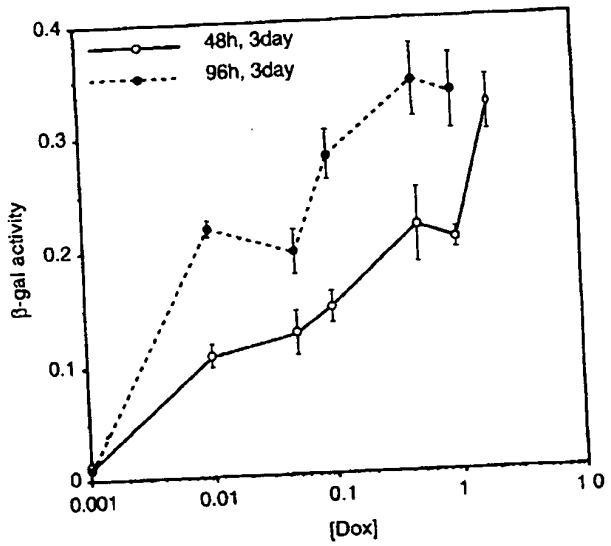

Fig. 4 Dose response of transgene induction by dox. Young adult progeny of cross rtTA(2)C1 × 7TAdh(2)A2 were fed the indicated concentrations of dox for 48 h and allowed to recover for 3 days (*open circles*), or for 96 h plus a 3-day recovery period (*closed circles*). β-Gal expression was quantitated as in Fig. 3

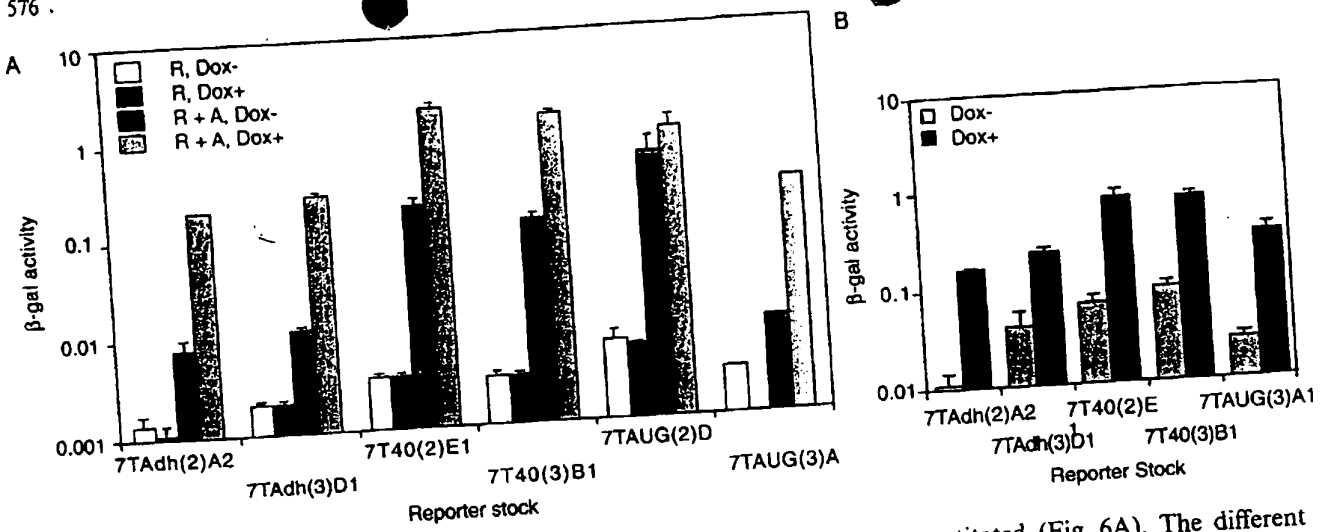

Fig. 5A, B Comparison of transgenic reporter constructs and lines. All dox treatments were for 48 h plus 3 days recovery. **A** Assay in young adults. The indicated reporter lines (R, reporter alone) were assayed with and without dox treatment, as indicated. Each indicated reporter line was also crossed to the rtTA line rtTA(2)C1, and the progeny (R + A, reporter plus activator) were assayed with and without dox treatment, as indicated. **B** Assay in old adults. rtTA line rtTA(2)C1 was crossed to each indicated reporter line, and old adult progeny were assayed with and without dox treatment, as indicated. β-Gal expression was quantitated as in Fig. 3

was observed with all three constructs, with induction factors ranging from 12- to 25- fold. In general, the 7T40 reporter construct gave higher levels of β-gal expression than the other two reporter constructs; however, the background expression in the absence of dox was also higher. Thus, the induction factor achieved was similar for each of the three reporter constructs.

To determine if the system functions during aging of *Drosophila*, the activity of each reporter construct was also assayed in senescent (30-day-old) flies (Fig 5B). Each reporter was found to support dox-induced β-gal expression in senescent flies, with induction factors ranging from 8- to 15-fold.

The dox-inducible system is dependent upon efficient, general expression of the transactivator construct, rtTA. Because the chromosomal site of insertion of the rtTA transgene can affect the level of expression, different independent rtTA transgenic lines may vary in their activity. To compare their activities, each of 13 independent rtTA transgenic lines was crossed to the 7TAdh(3)D1 reporter, and the efficiency of induction of β-gal by dox was quantitated (Fig. 6A). The different independent rtTA lines were found to vary in activity, both with regard to the amount of background β-gal activity in the absence of dox, and with regard to the maximum level of induction in the presence of dox. Transgenic transactivator line rtTA(3)E2 appeared to be the best: in the absence of dox, background β-gal levels were as low as in flies carrying the reporter construct in the absence of any transactivator, and dox treatment yielded a 40-fold induction. To confirm this result, each

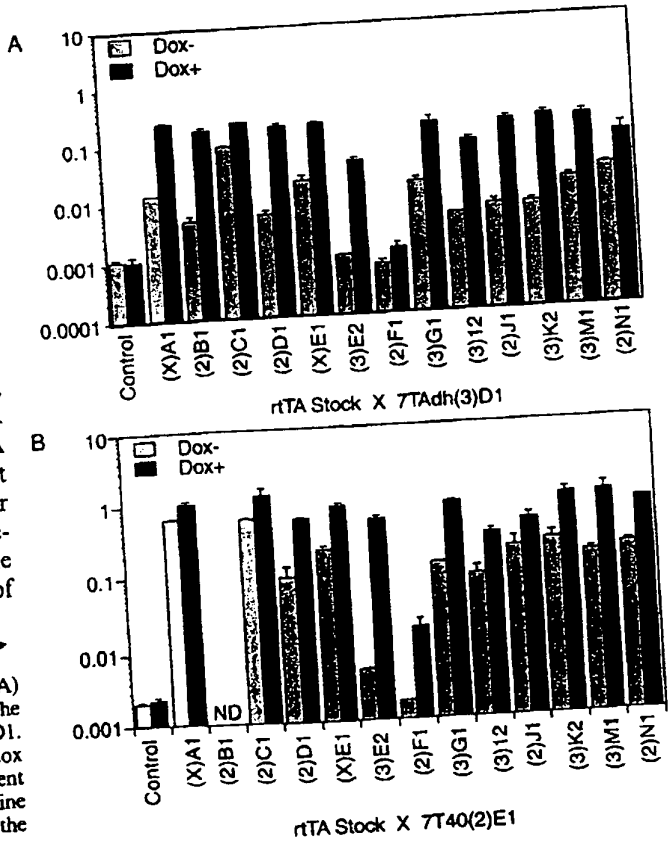

Fig. 6A, B Comparison of different transgenic transactivator (rtTA) lines. All dox treatments were for 48 h plus 3 days recovery. **A** The indicated rtTA lines were each crossed to reporter line 7TAdh(3)D1. The young adult progeny from each cross were assayed without dox treatment (Dox-; *stippled bars*), and with 1.0 mg/ml dox treatment (Dox+; *black bars*), as indicated. Control was the reporter line 7TAdh(3)D1 alone. **B** The experiment in A was repeated using the reporter line 7T40(2)E1. ND, not done

transactivator stock was also tested in combination with reporter stock 7T40(2)E1 (Fig. 6B). Again the various transactivator lines varied with regard to background and maximal level of induction, and their activity relative to each other was similar to that observed using the 7TAdh reporter. Line rtTA(3)E2 was again found to have the lowest background, and to be the most active, yielding 100-fold induction of β-gal in response to dox.

One potential use of the dox-inducible system in *Drosophila* is in the analysis of the effects of specific genes on the aging process. Ideally, for such experiments, the system itself should not have any effect on life span. To characterize the system for effects on life span, several genetic backgrounds were tested for longevity, with and without dox feeding. Wild-type flies exhibited no negative effects on life span when fed with 0.1 mg/ml dox (Fig. 7A). Double transgenic flies (rtTA(2)C1 × 7TAdh(2)A2) expressed high levels of β-gal in response to 0.1 mg/ml dox (Fig. 4), and this expression also had no detectable negative effects on lifespan (Fig. 7A). Finally, a different combination of transactivator and reporter were tested. Transactivator rtTA(X)A1 was crossed to reporter 7TAdh(3)D1 and age-synchronized cohorts of adult flies were treated throughout their adult lifespan with no dox, 0.01 mg/ml dox, or 1.0 mg/ml dox (Fig. 7B). Treatment with 1.0 mg/ml dox was found to have a negative affect on life span. However, treatment with 0.01 mg/ml dox had no detectable affect on life span. The same results were obtained with several other combinations of rtTA and reporter lines (data not shown). Since 0.01 mg/ml dox and 0.1 mg/ml dox allow high-level induction of β-gal (Fig. 4), and have no detectable effect on lifespan (Fig. 7), these results suggest that the system should be useful for assaying the effects on life span of overexpression of specific genes.

Discussion

The hybrid transcriptional activator (rtTA) consisting of the rtR fused to the transcriptional activation domain of the herpes virus protein VP16 has previously been shown to be capable of supporting dox-induced transcription in transgenic mice. The experiments presented

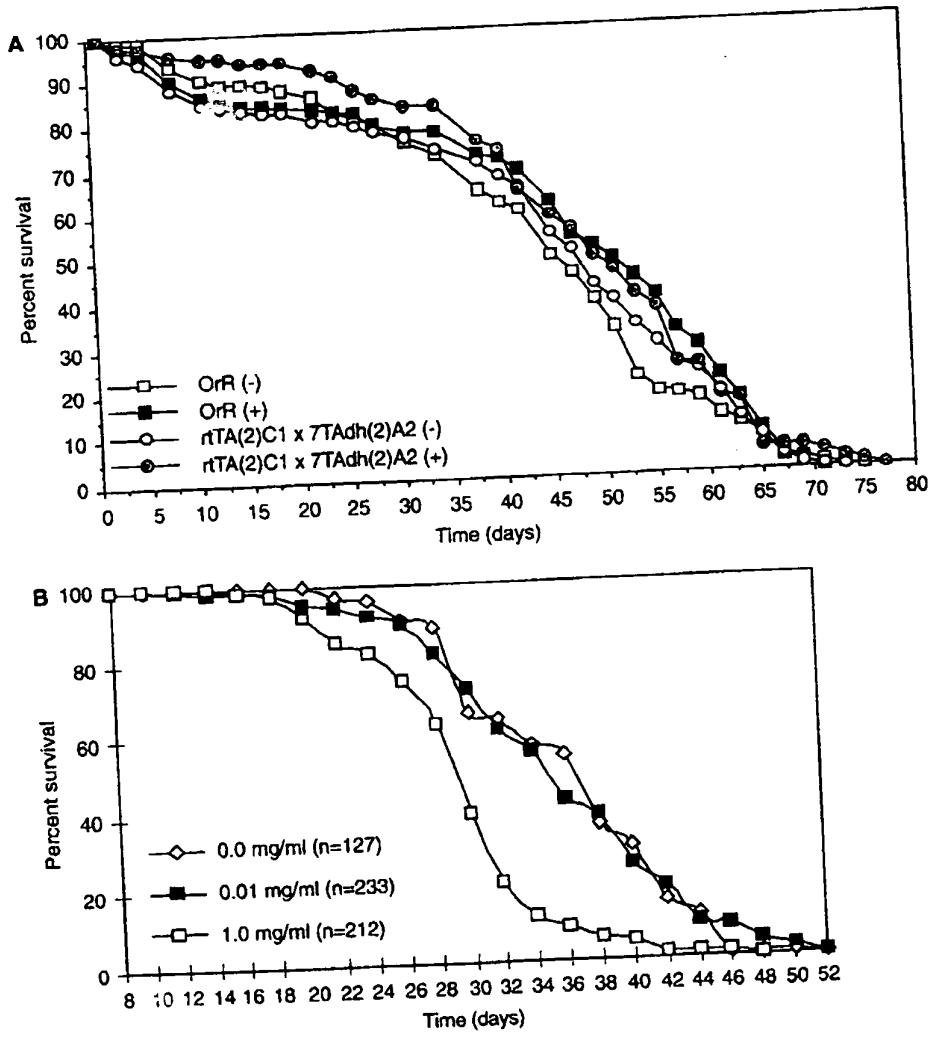

Fig. 7A, B Affect of dox-induced β-gal expression on *Drosophila* adult life span. **A** Wild-type Oregon R strain flies were treated throughout their adult lifespan either without dox (*open squares*) or with 0.1 mg/ml dox (*filled squares*), at 25° C. Flies were fed or mock-fed dox for 2 days, and then allowed to recover on standard media for 2 days, and this regimen was repeated until all the flies had died. The percentage of flies surviving is plotted as a function of time in days. The same experiment was performed using progeny from cross rtTA(2)C1 × 7TAdh(2)A2 grown in the absence of dox (*open circles*) or in the presence of 0.1 mg/ml dox (*closed circles*). At least 200 flies were used for each of the four survival curves. **B** Progeny from the cross rtTA(X)A1 × 7 TAdh(3)D1 were grown throughout their adult life span in the absence of dox (*open diamonds*), or in the presence of 0.01 mg/ml dox (*filled squares*), or 1.0 mg/ml dox (*open squares*), at 25° C. The number of flies used (n) for each survival curve is indicated. The different genotypes assayed in **A** and **B** vary in life span relative to each other, which is not unexpected due to the large effects of genetic background on life span (Curtsinger et al. 1995; Tower 1996)

here demonstrate that this rtTA functions in transgenic *Drosophila*, and can activate transcription at both the *hsp70* and *Adh* core promoters when they are linked to tetO sequences. Since all three reporter constructs performed similarly, the results suggest that in this system there is no significant difference in the effectiveness of the SV40 poly(A) signal relative to the *hsp70* poly(A) signal, and no significant difference between the effectivness of the *Adh*, *hsp70* and *Ubx* 5′ UTR regions. Dox-induced transgene expression was detected in all tissues, and induction ranged from 10- to 100-fold. Different transgenic lines containing the rtTA construct varied considerably in activity. Variation was observed in the maximal level of induction achieved, and in the amount of background activity observed in the absence of dox. This variation is likely to be due to chromosomal position effects on the expression of the rtTA transposon, and perhaps to other differences in the genetic background of the lines. The different transgenic lines of the reporter constructs also varied in activity, most probably for the same reasons. The maximum degree of induction that was achieved was 100-fold. This is dramatically less than the five orders of magnitude induction obtained with the tet-on system in transgenic mice (Kistner et al. 1996). The maximal induction achieved in *Drosophila* is limited by at least two factors: First, the reporter constructs are slightly leaky, in that variable, low-level β-gal activity is detected even in the absence of the rtTA transactivator. Second, the rtTA transactivator appears to be partially active even the absence of dox treatment, in that reporter plus transactivator was often more active than reporter alone. The first problem might be addressed by protecting the reporter constructs from position effects with insulator elements (Roseman et al. 1993), and/or by identifying a less leaky core promoter. The second problem can be mitigated by identifying particular rtTA lines, such as rtTA(3)E2, which exhibit less background activation. Finally, we hypothesize that the herpes virus VP16 transcriptional activation domain used to create the rtTA transactivator may be better suited to interaction with the mammalian transcriptional machinery than with the *Drosophila* transcriptional machinery. This possibility may also be relevant to the slower time course of induction observed in *Drosophila*.

Despite its limitations, this inducible system has several potential advantages relative to the use of heat shock gene promoters. The dox-inducible system should be useful for studying hsps, as it will allow the investigator to induce the expression of a single hsp, and potentially inhibit its expression with antisense RNA, without inducing the endogenous heat shock response. The dox- inducible system also allows the investigator to induce a gene of interest at any time during the life cycle. This is particularly relevant to study of the aging process (Curtsinger et al. 1995; Tower 1996), where it is often desirable to alter gene expression specifically in the adult. For example, constitutive over-expression of Cu/Zn SOD may have beneficial effects on *Drosophila* life span but it also appears to have toxic affects during pupal development (Reveillaud et al. 1991). Using the dox-inducible system it should be possible to avoid toxic effects during development and cause over-expression of transgenes specifically in the adult where beneficial effects on life span may be more apparent.

The dox-inducible system should be readily adaptable to tissue-specific induction. Replacement of the constitutive *Actin5C* promoter in the transactivator construct rtTA with a tissue-specific promoter should provide tissue-specific expression of the rtTA transactivator and thus tissue-specific induction of the reporter. An elegant system for tissue-specific expression of the yeast GAL4 transactivator has been developed for *Drosophila* (Brand and Perrimon 1993; Brand and Dormand 1995). In this case tissue-specific expression of the GAL4 transactivator is driven by an "enhancer-trap" system: the transactivator is under the control of a weak transcriptional promoter which can become activated in a tissue- and temporal-specific manner when the P element inserts near transcriptional enhancer sequences in the chromosome. The large variety of tissue- and temporal-specific GAL4 transactivator expression patterns generated thus allows tissue- and temporal-specific expression of "reporter" type constructs containing GAL4 binding sites in their promoters. This system could be adapted to drive expression of the rtTA transactivator, thus creating a large variety of tissue-specific expression patterns inducible by dox.

Finally, it may be possible to create dox-dependent mutations in *Drosophila*. P element constructs with transcriptional promoters directed out of the end of the P element can cause over-expression and/or mis-expression of genes near the site of insertion, sometimes causing dominant mutations (Rorth 1996; Hay et al. 1997). Creation of a P element with a dox-inducible promoter directed out of the P element into flanking DNA sequences should sometimes cause dox-induced over-expression of a gene near the insertion site. This method should thus yield conditional (dox-dependent), dominant, gain-of-function mutations which would be useful for many types of genetic analyses; such experiments are now underway.

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Functional Characterization of the Enhancer Blocking Element of the Sea Urchin Early Histone Gene Cluster Reveals Insulator Properties and Three Essential *cis*-acting Sequences

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Insulator elements can be functionally identified by their ability to shield promoters from regulators in a position-dependent manner or their ability to protect adjacent transgenes from position effects. We have previously reported the identification of a 265 bp *sns* DNA fragment at the 3′ end of the sea urchin H2A early histone gene that blocked expression of a reporter gene in transgenic embryos when placed between the enhancer and the promoter. Here we show that *sns* interferes with enhancer-promoter interaction in a directional manner. When *sns* is placed between the H2A modulator and the inducible *tet* operator, the modulator is barred from interaction with the basal promoter. However, the *tet* activator (tTA) can still activate the promoter, even in the presence of *sns*, demonstrating that *sns* does not interfere with activity of a downstream enhancer. In addition, the H2A modulator can still drive expression of a divergently oriented transcription unit, suggesting that *sns* does not inhibit binding of transcription factor(s) to the enhancer. To identify *cis*-acting sequence elements within *sns* which are responsible for insulator activity, we have performed *in vitro* DNase I footprinting and EMSA analysis, and *in vivo* functional assays by microinjection into sea urchin embryos. We have identified three binding sites for protein complexes: a palindrome, a direct repeat, and a C + T sequence that corresponds to seven GAGA motifs on the transcribed strand. Insulator function requires all three *cis*-acting elements. Based on these results, we conclude that *sns* displays properties similar to the best characterized insulators and suggest that directional blocking of enhancer-activated transcription by *sns* depends on the assembly of distinct DNA-protein complexes.

Keywords: histone genes; enhancer blocking; insulator; H2A enhancer; microinjection

Introduction

Proper temporal and spatial regulation of gene expression requires the orderly and efficient interaction of transcription factors with their cognate sites. The elucidation of how this might occur is one of the major challenges in molecular biology. If transcription units are organized into independent functional domains, enhancers could activate transcription from a promoter within the same domain but would be restrained from interacting with promoters in external domains. Insulators seem to be involved in the organization of the eukaryotic genome into domains of gene expression.[1–4] Insulators have been identified because they interfere with

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Abbreviation used: EMSA, electrophoretic mobility shift assay.

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enhancer-promoter communication only when positioned between them; for this reason they are generally called enhancer blocking elements. Although mechanisms by which these elements function in the normal context are not fully understood, it is thought that binding of chromosomal proteins to specific insulator sequences and subsequent protein-protein interactions can directionally restrict enhancer activity (for recent reviews see:[5,6]). The best characterized of these enhancer blocking elements are the *Drosphila*, scs, scs' and gypsy elements,[7-10] and the chicken HS4 insulator.[11,12] In addition to enhancer blocking activity, insulators can protect a transgene from chromosomal position effects when placed in flanking positions. However, only the chicken HS4[13,14] and the *Drosphila*, gypsy[15] elements seem to confer a barrier which prevents heterochromatin from spreading. Gypsy elements can also buffer a promoter from the silencing effect of the polycomb responsive element (PRE).[16] Elements which counteract silencing and act as heterochromatin boundaries have also been identified in yeast.[17-19] However, it is not known whether the yeast elements can also act as insulators of enhancer activity or display only chromatin boundary functions.[20]

Insulator DNA elements seem to be present in the tandemly repeated sea urchin early histone genes. The five early histone genes are expressed coordinately after meiotic maturation of the egg, and in early cleavage embryos until the blastula stage.[21] Although there is a single enhancer within the histone repeat unit, the 30 bp modulator of the H2A gene[22,23] each gene within the repeat is apparently regulated by gene-specific transcriptional elements.[24-26] From this observation, we hypothesized that there might be chromosomal elements which would direct and restrict H2A modulator function to its cognate H2A promoter. Subsequently, we identified a 265 bp sequence at the 3' end of the H2A early histone gene (see map in Figure 1), that, in microinjected sea urchin embryos, showed a blocking activity when placed between the enhancer and the promoter. We termed this sequence silencing nucleoprotein structure (*sns*). Although proof of the directionality of enhancer blocking activity was lacking, experimental evidence suggested that *sns* is an insulator of enhancer function rather than a general repressor of transcription. *sns* blocked enhancer-promoter interaction only when interposed, in either orientation, between a multiple array of the H2A modulator/enhancer and the basal thymidine kinase (tk) promoter. No other position influenced transgene expression. Furthermore, *sns* interfered with enhancer function but not with the activity of the basal promoter, in that it mantained the capacity to silence transgene expression when it was placed at a distance of 2.7 kb from the promoter.[28] Interestingly, our results suggested evolutionary conservation of enhancer blocking mechanisms, as *sns* was able to shield promoters from viral enhancers in transient and stably transfected human cell lines, as well as in *Xenopus laevis* oocytes[28] (unpublished observations).

Figure 1. Map of the H2A transcription unit. CCAAT (CBF), modulator (MBF), putative GAGA factor binding sites in the H2A promoter, and the transcription start site are indicated. The *sns* fragment spanning from the *Hpa*II to the *Sau*3AI restriction sites starts 14 nucleotides upstream of the stop codon of the H2A gene and resides in a region where three micrococcal nuclease hypersensitive sites (vertical thick arrows) appear at gastrula stages.[27]

Here we have further characterized the properties of the sea urchin *sns* DNA fragment. We present evidence demonstrating that *sns*, as in the case of the best characterized insulators, affects only enhancers located distally from the promoter and displays a directionality in enhancer blocking activity. In addition, we report the identification of three protein binding sites within *sns* that, as demonstrated by functional assays in transgenic sea urchin embryos, are collectively required for insulator activity.

Results

sns interferes with enhancer function in a directional manner

Utilizing RNase protection assays, we previously demonstrated the ability of *sns* to inhibit enhancer-activated expression of a transgene in microinjected sea urchin embryos only when interposed between an array of the H2A modulator/enhancer and the *tk* promoter.[28] Because basal expression from *tk* or other viral promoters in sea urchin is negligible, effects on transcription were dramatic, with previously abundant transcript levels becoming undetectable. We have used the same approach to investigate whether *sns* evinces other behaviors expected of insulators. We placed *sns* between two enhancers in a chloramphenicol-acetyl transferase (CAT) gene transcription unit (Figure 2(a), construct 2). As a distal enhancer, we used the sea urchin H2A modulator array, containing at least four binding sites for the MBF-1 *trans*-activator; the proximal enhancer was the heptamerized tetracycline (*tet*) operator. In HeLa cells, the *tet* operator is induced upon binding of the Tet repressor-VP16 activation domain chimera (tTA *trans*-activator) and stimulates transcription of a reporter gene by several orders of magnitude.[29] The *tTA* gene was placed under the control of the multiple modulator elements and the *tk* minimal promoter (construct A). This construct was coinjected with the CAT reporter gene driven by the *tet*

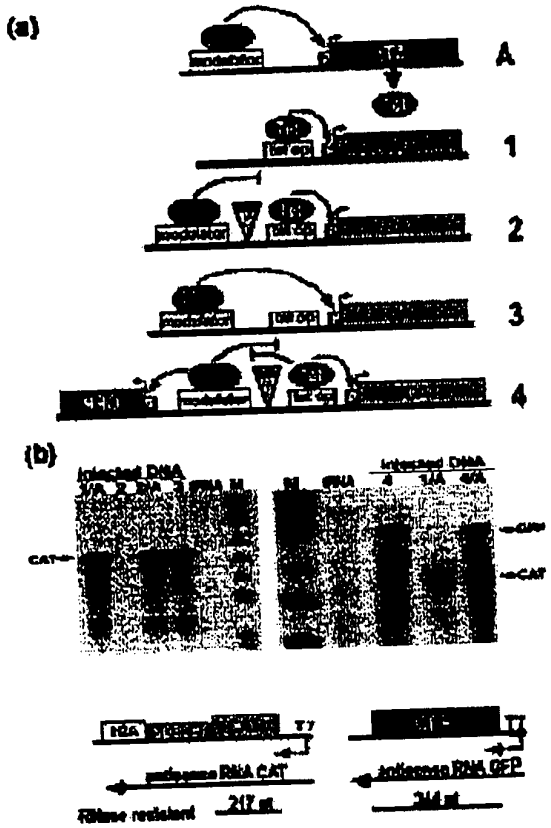

Figure 2. Polar and directional effects of *sns* on enhancer-promoter interaction. (a) Schematic representation of the microinjected plasmids. Binding of the MBF and the tTA transactivators, respectively, to the modulator and *tet* operator arrays is also indicated. Curved arrows refer to the activation of promoter by the bound factor; small arrows point to the transcription start site. (b) Total RNAs from microinjected embryos were processed to detect enhancer-activated transgene expression by RNase protection using antisense ^{32}P-labelled probes transcribed *in vitro* from the constructs illustrated below the gel images. Arrows point to the 217 nucleotide CAT and 344 nucleotide GFP RNase-resistant fragments. tRNA was used as negative RNA control. The RNase digestion products and end labelled *Hpa*II-digested pBluescript DNA (M) were run on denaturing polyacrylamide gels. Coinjection of constructs 1 and A (lanes 1/A) leads to CAT expression. In the absence of tTA, construct 2 is silent because the modulator is blocked by *sns* (lane 2). Coinjection of constructs 2 and A (lane 2/A) *trans*-activates *CAT* gene expression. Construct 3 expresses the *CAT* transgene because the *tet* operator does not interfere with the modulator (lane 3). Construct 4, in the absence of tTA, expresses only the GFP transgene (lane 4); if coinjected with A both the *CAT* and the *GFP* reporter genes are *trans*-activated (lane 4/A). Hence, *sns* does not block the binding of the transcription factors to the enhancers.

operator (construct 1). We predicted that expression of the tTA would elicit *trans*-activation of the transgene. As shown in Figure 2(b) (lane 1/A) this was indeed the case. Next, we tested the constructs with two enhancers. As expected, construct 2 containing *sns* between the MBF-1 and tTA binding sites was transcriptionally silent (lane 2), indicating that *sns* blocked the *trans*-activating function of the MBF-1 and that the *tet* operator was inactive in the absence of tTA. When the activator expression plasmid (construct A) was coinjected with construct 2, *trans*-activation of the transgene occurred (lane 2/A). The intensity of the CAT mRNA band detected in embryos injected with the two different plasmid combinations was almost identical (compare lane 1/A and 2/A), suggesting that neither the modulator nor *sns* sequences affected the extent of activation by tTA. In addition, the *tet* operator sequences did not interfere with the enhancer activity of the modulator, as similar levels of transgene expression were detected in embryos injected with construct 3 (lane 3). In summary, these experiments strongly suggest that *sns*, like chromatin insulators, has the ability to block the distal enhancer from communicating with the promoter but has no influence on the proximal one, when situated between the two.

We also investigated *sns* behavior in the context of a bidirectional transcription construct. A construct was made in which the modulator array and *tet* operator direct expression of two divergently transcribed reporter genes, encoding either CAT or green fluorescent protein (GFP). The *sns* sequence was inserted between the modulator and *tet* operator (construct 4 in Figure 2(a)). RNase protection assays were performed with RNA extracted from transgenic embryos, utilizing probes for both CAT and GFP in the same hybridization reaction. Only *CAT* mRNA was detected in embryos microinjected with constructs 1 and A (Figure 2(b), right panel, lane 1/A). As expected, in the absence of the tTA activator, the *CAT* transgene was not expressed in embryos injected with the bidirectional transcription unit (lane 4), presumably because *sns* interrupted the interactions between MBF-1 and the basal transcription apparatus. However, *sns* did not restrain MBF-1 from activating the divergent *GFP* transcription unit (lane 4). Subsequently, expression of tTA allowed for *trans*-activation of the *CAT* transcription unit (lane 4/A). From these results we conclude that *sns* blocks enhancer activity in a directional manner.

In vitro binding of proteins to *sns* sequences

To identify nuclear protein binding sites within *sns*, we performed DNase I footprint analysis and electrophoretic mobility shift assays (EMSA) with nuclear extracts from gastrula stage embryos. Two DNase I protected regions, defined as Box A and Box B, were mapped to both strands in the 5′ half of *sns* (Figure 3). The specificity of protein-DNA interaction was assessed by oligonucleotide compe-

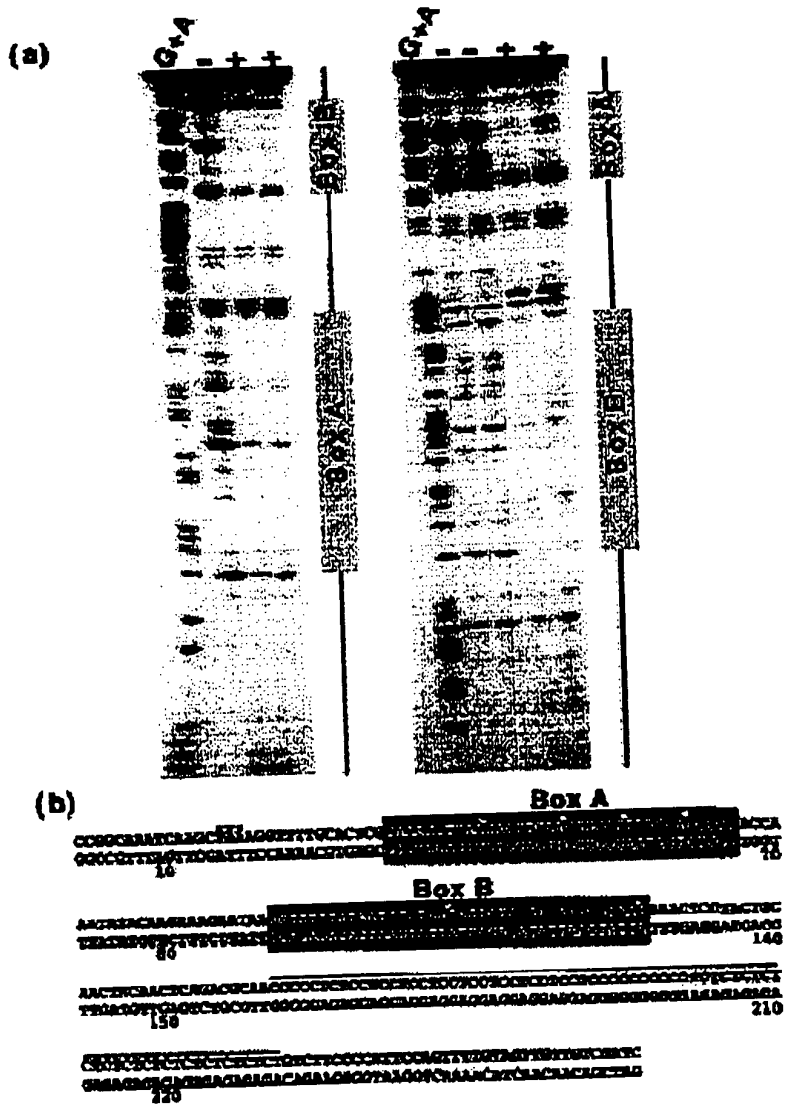

Figure 3. DNase I footprinting of an end-labelled *sns* sub-fragment spanning from nucleotides 2 to 157. (a) Sense strand (left) and antisense strand (right) were incubated with either BSA (lanes-) or nuclear extracts from gastrula stage embryos and digested with 5 μg of DNase I for three to five minutes on ice (lanes+). Digestion products were analyzed together with the cleavage products of the G + A sequence reaction on denaturing polyacrylamide gels. (b) Nucleotide sequence of the *sns* element. Asterisks mark the stop codon of the H2A gene. Sequences of protected Box A and Box B are in black boxes. Arrows inside boxes point to the Box A inverted and direct repeats and to the Box B direct repeat. Pyrimidine stretches are overlined.

tition experiments in EMSA. Figure 4 shows that both DNA-protein complexes were suppressed by an excess of unlabelled homologous probe, while they were not affected by an excess of unlabelled heterologous sequences. As indicated in the sequence shown in Figure 3(b) and in the drawing of Figure 4, Box A contains two notable sequence features: a C + A perfect direct repeat (DR), and immediately downstream the palindrome (IR) which is one of the *cis*-acting elements involved in 3′ RNA processing.[30] Because the IR sequence alone competed as efficiently as the entire Box A, we conclude that the palindrome is the protein binding site within Box A.

To search for further protein binding sites, we analyzed the pyrimidine stretch (C + T) at the 3′ end of *sns* sequences. This fragment contains 14 TC repeats that in the bottom strand correspond to seven GAGA sequences. EMSA analysis with nuclear extracts demonstrated that the C + T rich fragment formed a predominant DNA-protein complex that was specifically competed by an

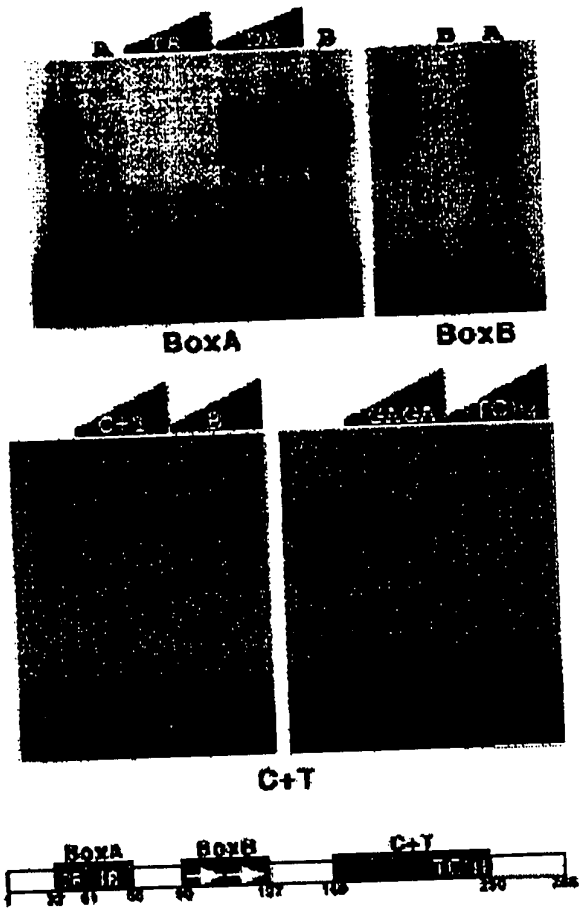

Figure 4. EMSA analysis of nuclear protein binding sites within *sns*. The three end labelled probes Box A, Box B and C + T, are underlined in the schematic drawing of the *sns* fragment. DR and IR refer, respectively, to the direct repeat and palindrome of Box A; white arrows in the Box B indicate a tandem repetition; (TC)14 refers to the 14 repetitions of the TC doublet. The C + T fragment was obtained by PCR amplification. All other probes were obtained by annealing complementary oligonucleotides. In competition experiments, nuclear extracts were preincubated with an excess of unlabelled homologous or heterologous probes prior to the addition of 1 ng of the labelled probe. The amounts used were: 100 ng for Box A (A) and Box B (B); 50 ng and 100 ng for IR, DR, C + T, GAGA (the GA repeats located upstream the H2A modulator), and (TC)14. The DNA-protein complexes were resolved by polyacrylamide gel electrophoresis.

excess of the homologous fragment (Figure 4). Of particular significance, protein binding was also specifically competed when nuclear extracts were pre-incubated either with an excess of a sequence containing four GAGA repeats, which is located upstream of the *H2A* modulator (see Figure 1), or with an oligonucleotide containing the 14 TC dinucleotides found at the 3′ end of the pyrimidine region (see sequence in Figure 3). The former competition was slightly less efficient, perhaps due to the presence of fewer (eight) TC dinucleotide repeats. These observations demonstrate the binding of nuclear protein(s) to GAGA sequences in sea urchin and suggest that a putative GAGA factor might contribute to the enhancer blocking function of *sns*.

Deletion of either the Box A palindrome or the 3′ CT repeats abolishes *sns* insulator function

We used the enhancer blocking assay to test the effect of 5′ and 3′ deletions of the *sns* fragment on the expression of a transgene driven by the *H2A* modulator in transgenic sea urchin embryos. The *sns* deletion mutants shown in Figure 5(a) were cloned between multiple copies of the 30 bp modulator/enhancer of the H2A histone gene and the tk promoter of the M30-CAT reporter plasmid (Figure 5(b)). Resulting constructs were microinjected into sea urchin eggs, embryos raised till gastrula stages and processed to determine *CAT* transgene expression by RNase protection analysis. Results depicted in Figure 5(c) are representative of several microinjection experiments. In agreement with our previous reports,[23,31] in the presence of one or several copies of the 30 bp histone H2A modulator sequence, transcriptional activation from the tk promoter occurs efficiently, as evidenced by abundant transgene transcripts (Figure 5(c), lanes 3, 9, 11). These M30-CAT constructs demonstrated once again the enhancer blocking function of the intact *sns* (lane 5). Deletions from either the 5′ or the 3′ that remove Box A(Δ sns), or the pyrimidine rich sequence (ΔIII sns), respectively, impaired the blocking activity of *sns* (lanes 4 and 13). In fact, levels of CAT transcripts were comparable to the construct lacking *sns* (lanes 3 and 11). As expected, 5′ deletions that

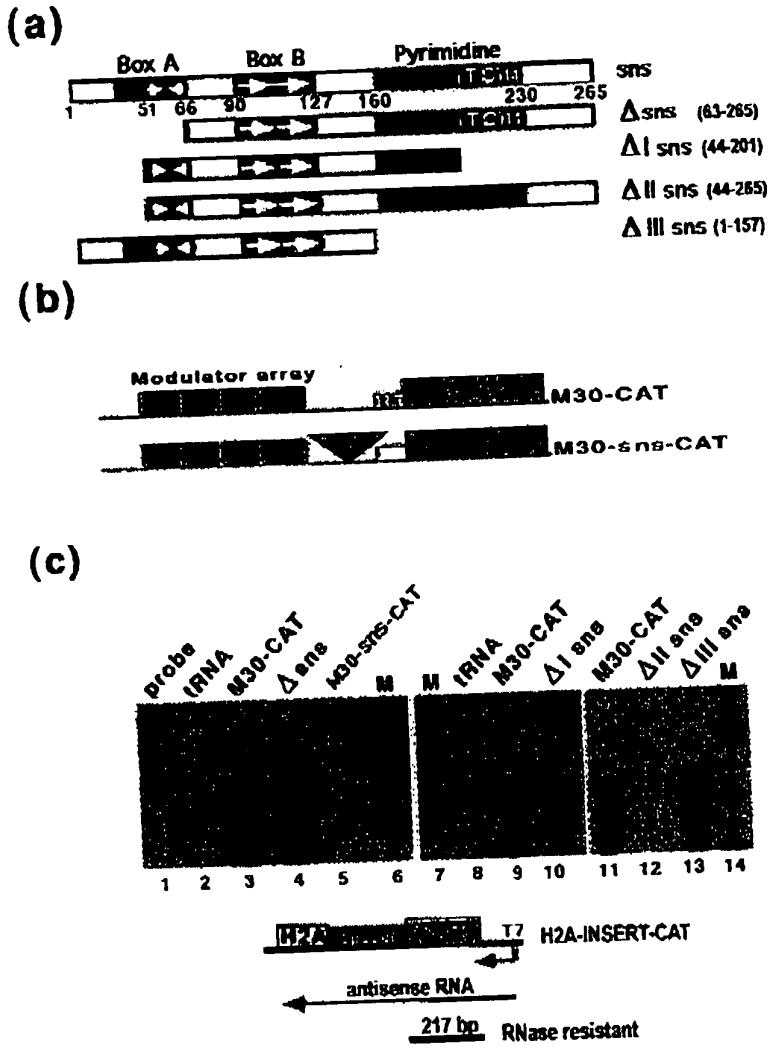

Figure 5. Functional activity of the *sns* deletion fragments. (a) Maps of *sns* and of the different deletion fragments assayed for enhancer blocking activity. (b) Schematic drawing of the microinjected plasmids. *sns* or the *sns* deletion fragments were inserted between the H2A modulator array and the tk promoter of M30-CAT to generate the M30-sns-CAT plasmids. (c) A ^{32}P-labelled antisense CAT RNA (lane 1), transcribed *in vitro* from the H2A-INSERT-CAT was used to perform RNase protection assays on microinjected embryos at gastrula stage. Electrophoretic analysis of the RNase digestion products was carried out on denaturing polyacrylamide gels. Lanes 3, 9 and 11: microinjection of the positive control M30-CAT to monitor enhancer-activated expression of the transgene. Lane 5: microinjection of M30-sns-CAT to monitor enhancer blocking activity of *sns*. Lanes 4, 10, 12 and 13: microinjection of reporter plasmids carrying, respectively, Δsns, ΔI sns, ΔII sns and ΔIII sns; only ΔII sns maintained the ability to attenuate the enhancer. Lanes 2 and 8: tRNA negative control. Lanes 6, 7 and 14: pBluescript *Hpa*II-digested end-labelled DNA markers.

left the palindrome intact, (ΔII sns) exhibited wild-type *sns* enhancer blocking activity (lane 12). Finally, removal of the TC repeats (ΔI sns) from ΔII *sns*, abolished the ability of *sns* to affect enhancer-promoter interaction (lane 10). Altogether, these results are consistent with the nuclear protein binding sites defined above and indicate that the Box A palindrome and the GAGA sites are essential for *sns* to block communication between the modulator and the tk promoter.

Box B is also essential for enhancer blocking activity

The experiments described in the previous sections suggest that the enhancer blocking function of *sns* relies on the assembly of protein complexes

at the Box A palindrome and at the GAGA sites. Because binding of proteins to Box B was also detected, we investigated whether these interactions were also essential for *sns* activity. Toward this end, we performed an *in vivo* competition experiment. We have previously used this approach to demonstrate that binding of the MBF-1 transcription factor to the modulator is required for activation of a transgene driven by the histone H2A promoter.[31] As indicated in Figure 6, sea urchin embryos were injected with the *sns*-containing transgene construct together with increasing amounts of ligated oligonucleotides containing either Box B (lanes 3, 4) or the Box A (lane 6) sequences. As levels of enhancer-activated transgene transcripts were similar to those seen with M30-CAT plasmid (lane 5), these results demonstrate that either oligonucleotide prevented enhancer blocking (lanes 2,7). Hence, titration of either Box A or Box B binding proteins by injecting their target sites impaired the ability of *sns* to block enhancer-promoter interaction.

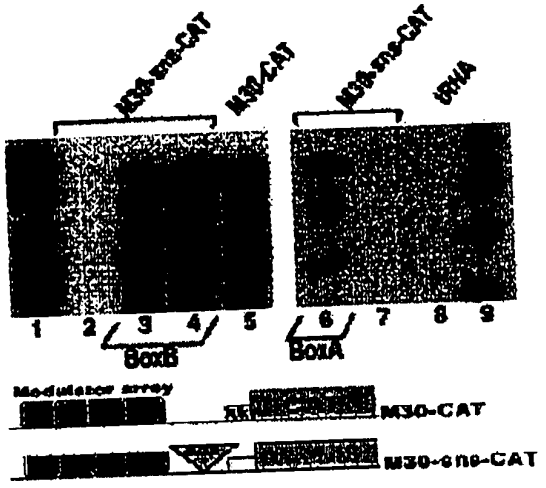

Figure 6. *In vivo* competition of *sns* function. Transgenic embryos were obtained by microinjecting the constructs drawn below the fluorograph with or without an excess of either BoxB or BoxA oligonucleotides. Black boxes represent the modulator array, the large shaded triangle the *sns* fragment. RNase protection experiments were carried out with total RNA as described in the legend to Figure 5. Lanes 2 and 7: injection of M30-sns-CAT; the enhancer is blocked. Lanes 3, 4 and 6: co-injection of M30-sns-CAT and 40-fold (lane 3) or 70-fold (lane 4) excess of ligated BoxB oligonucleotide or 70-fold (lane 6) excess of ligated BoxA oligonucleotide relieves the block. Lane 5: injection of M30-CAT; enhancer activity of the modulator array. Lanes 1 and 9 show relevant bands of DNA markers.

Discussion

Insulators are a new class of genetic elements that can modulate the activity of enhancer or other regulative sequences.[3,5] The few elements identified principally in *Drosophila* and chicken display two important characteristics: polarity and directionality of the effects of insulation of enhancer activity.[1,2] The former signifies that only enhancers located distally from the promoter with respect to the site of insertion of the insulator are attenuated in the interaction with the promoter. The second feature is that insulators do not prevent a blocked enhancer from activating transcription from a divergent promoter.[32,33] Consistently, we have shown that *sns* when placed between two enhancers, insulated the promoter-distal modulator without affecting the function of the downstream *tet* operator. In addition, *sns* did not interfere with the *trans*-activating capacity of the modulator in the other direction. Taken together, these results rule out that insertion of *sns* between enhancer and promoter represses enhancer-promoter interaction by enhancer inactivation, for example by inducing local assembly of a repressive chromatin structure.

As first shown in *Drosophila*, the directional enhancer blocking activity of insulator elements depends on the assembly of specific DNA-protein complexes. The gypsy insulator is perhaps the best-studied system with respect to the characterization of protein components that interact with insulator DNA. One of these components, the suppressor of Hairy-wing [su(Hw)] protein, binds to a reiterated target sequence[34] and recruits the second component, the mod(mdg4) protein[35] that displays properties characteristic of trithorax-group (trxG) genes.[36] The BEAF protein binds to the *scs*′ insulator[37] which characterizes a class of chromosomal elements found at many loci.[38] Interestingly, seven tandem copies of an oligonucleotide containing BEAF binding sites has partial enhancer blocking activity.[37] The capability of the chicken HS4 insulator to interfere with enhancer-promoter interaction resides in a 42 bp fragment that contains a binding site for the CTCF transcription factor.[39] Binding of CTCF occurs also to several vertebrate insulators and to the unmethylated ICR (imprinting-control region) that displays enhancer blocking activity to control imprinted expression of the Igf2 gene.[40,41] Therefore, it is not surprising that the directional enhancer blocking activity of *sns* depends on specific DNA-protein interactions. It is of some interest that, while the enhancer blocking capacity of the *gypsy*, *HS4*, and to some extent the *scs*′, insulators relies on the recognition of a single or a reiterated binding site, *sns* contains three different *cis*-acting elements. Our results strongly suggest that all of these are needed to prevent enhancer-promoter interaction. In fact, deletion of either the Box A palindrome or the 14 TC repeats completely impaired *sns* function. Furthermore, microinjection of excess Box A or Box B and very recently GAGA (not shown) binding sites relieved the inhibition of the modulator in the *sns*-containing constructs. The most plausible explanation of the *in vivo* competition results is that the excess of

binding sites titrated, either directly or indirectly, the factors responsible for the enhancer blocking activity. Based on these observations, we speculate that *sns* achieves directional enhancer blocking activity by cooperative interactions between all three different DNA binding proteins or protein complexes.

Our results demonstrate that, within Box A, only the palindrome is required for enhancer blocking activity. Deletion of the 5′ most direct repeats, upstream of the palindrome, does not impair *sns* function. In agreement with this observation, oligonucleotides containing the direct repeats failed to compete for binding of factors to Box A and did not form specific protein-DNA complexes (not shown). Of some interest, the palindrome forms a stem-loop RNA structure, highly conserved among the non-polyadenylated histone mRNAs, from sea urchin, and *Drosophila* to mammals and represents one of the signals recognized by 3′ pre-histone mRNA processing machinery.[30]

A second *cis*-acting element was identified within the pyrimidine tract that contains seven GAGA repeats in the inverted orientation. Based upon EMSA analysis, specific protein interactions occur at the GAGA sites of *sns* and presumably at GAGA sites located upstream of the H2A modulator. Because the enhancer blocking activity of *sns* is independent of orientation,[28] it is reasonable to assume that protein(s) related to a *Drosophila* factor which binds GAGA sequences might be involved in the mechanism that interrupts the interaction between enhancer and promoter in sea urchin. *Drosophila* GAGA factor is a DNA binding protein involved in chromatin remodelling processes.[42] GAGA factor alleviates, in combination with NURF, the repressive effect of chromatin[43] and participates in the assembly of the silencing Polycomb proteins at PRE.[44] Interestingly, binding of factors to GAGA sites occurs in the spacer between the *Drosophila* H3 and H4 histone genes,[45] and recent evidence indicates a direct involvement of GAGA factor in insulator activity. GAGA factor binding sites, found at the PRE adjacent to the Fab-7 insulator, cooperate with Fab-7 to maintain the specific parasegment domain of expression of the Abdominal-B gene.[46,47] In addition, mutation of GAGA sequences within the insulator of the even-skipped locus affects directional blocking of the iab-5 enhancer.[48] Despite the similarity of the binding site and the apparent involvement in insulator function, the sea urchin protein differs from the *Drosophila* GAGA factor because a *Drosophila* polyclonal anti-GAGA factor antibody failed to supershift the *in vitro* assembled nuclear protein-DNA complex from sea urchin (not shown). The cloning of the sea urchin GAGA factor encoding gene should clarify whether the *Drosophila* and sea urchin factors are evolutionary and functionally related. One working hypothesis, currently under investigation, is that interactions between the proteins of *sns* and the proteins bound to the GAGA sites of the H2A promoter, prevent the H2A enhancer from acting promiscuously to activate transcription of heterologous early histone promoters.

With the exception of the GAGA element, the *sns* insulator sequence motifs are distinct from those described for other insulators. However, there is some evidence to suggest that these insulator sequences and their binding factors are also evolutionarily conserved. Very similar sequences are present in equivalent positions in the histone H2A transcription unit of the sea urchin *Psammechinus miliaris* (not shown). In addition, we have recently found that *sns* can insulate a viral enhancer upon stable integration in human chromatin (unpublished) and that at least two of the identified *cis*-acting insulator sequence elements, Box B and TC dinucleotide repeats (Box A did not show DNA binding activity in our conditions), interact specifically with human nuclear proteins of two different cell types (unpublished results).

In conclusion, we have extended our previous characterization of the *sns* element by the demonstration that *sns* acts equivalently to previously well-characterized insulators in a number of ways. We have now identified *cis*-acting sequences required for directional enhancer blocking activity, which may be evolutionarily conserved, and include novel sequences. Our studies have significant implications both for the control of early histone gene regulation in sea urchins, and for more general mechanisms of insulator action. In addition, these sequences may prove to have practical applicability in genetic engineering situations where insulator action might be beneficial.

Materials and Methods

Construction of plasmids

Plasmids, schematically drawn in Figure 2, were obtained as follows. Plasmid A that expresses the tTA activator, was constructed by the substitution of the CMV promoter of the pUHD 15.1 vector[29] with a fragment containing an array of the modulator sequences and the *tk* promoter. The pUHD 15.1 plasmid was digested with *Xho*I and *Xba*I simultaneously, filled in and ligated with a blunt ended DNA fragment containing the modulator sequences. Plasmid 1 that expresses the *CAT* gene under the control of the *tet* operator and the CMV promoter, was constructed by cloning the *tet* operator and the CMV promoter from the pUHD 10.3 plasmid[49] into the *Xho*I restriction site of the pBL-CAT3 vector.[50] Plasmid 3 that expresses the *CAT* gene under the control of two enhancers, the *tet* operator and the modulator, was obtained by cloning the 180 bp *Hin*dIII-*Xba*I DNA fragment containing the modulator repeats, derived from M30-CAT, in the *Hin*dIII-*Xba*I-digested plasmid 1. In construct 2, the *Hin*dIII-*Xba*I DNA fragment containing *sns* was cloned into plasmid 3 between *tet* operator and the modulator sequences. The EGFP gene (Clontech) fused to the *tk* promoter was cloned in inverted orientation upstream the modulator of plasmid 2, to generate the construct containing the two divergent transcription units (plasmid 4). To generate the plasmid M30-CAT an array of the H2A modulator/enhancer sequences was cloned into the *Sal*I site upstream of the

tk promoter of the pBL-CAT3 vector. To obtain the plasmid M30-sns-CAT, *sns* was inserted into the *Xba*I site of M30-CAT, between the modulator and the *tk* promoter. The *sns* fragment was isolated by *Hpa*II and *Sau*3A restriction enzyme digestion of the *Sac*I subclone of the PH70 histone DNA.[21] The Δsns, ΔI sns, ΔII sns and ΔIII sns deletion fragments were obtained from *sns* by restriction enzyme digestion or PCR amplification. These fragments were cloned respectively in the *Xba*I and *Bam*HI restriction sites located in the polylinker between the modulator and the tk promoter of the M30-CAT. The plasmids used for the RNase protection experiments, H2A-INSERT-CAT and GFP, were obtained as follows. The former contains a 258 bp pUC fragment inserted between the H2A promoter and a CAT coding subregion as described.[29] The latter bears a 344 bp fragment of the EGFP gene.

DNase I protection and electrophoretic mobility shift assays

Sense and antisense *sns* end-labelled fragments, spanning the region between nucleotides 2 and 157, were obtained by PCR. BSA or nuclear extracts from *P. lividus* embryos, prepared as described,[51] were preincubated with 10 μg of poly(dA-dT)·(dA-dT) in 50 μl of 10 mM Hepes (pH 7.9), 60 mM KCl, 1 mM DTT, 1 mM EDTA, 8 % (v/v) glycerol for five minutes before the addition of the labelled fragments. After 20 minutes incubation, samples were digested with 5 μg of DNase I in 2.5 mM $MgCl_2$ for three to five minutes at 4 °C, phenol extracted and loaded on 8% (w/v) denaturing polyacrylamide gels. In EMSA experiments, three different *sns* probes were assayed for binding activity *in vitro*: Box A, Box B double-stranded oligonucleotides (see next paragraph for sequences) and the C + T containing subfragment (spanning nucleotides 137 and 262) obtained by PCR. 1 ng of end labelled Box A , Box B and C + T probes were incubated, for 30 minutes on ice, with 5 μg of nuclear extracts from sea urchin embryos at gastrula stage and 2 μg of poly(dI-dC)·(dI-dC) in 20 μl of 10 mM Hepes (pH 7.9), 60 mM KCl, 1 mM DTT, 1 mM EDTA, 4% Ficoll. In the competition experiments, unlabelled homologous or heterologous probes in the amounts described in the legend to Figure 4 were added to the preincubation mixture prior to the addition of the extracts. Binding reactions were run on non-denaturing polyacrylamide gels in 50 mM Tris, 50 mM H_3BO_3 and 2 mM EDTA (pH 8.3).

List of oligonucleotides

Only the sense strand is reported. Residues of the following oligos refer to the first nucleotide of the *sns* sequence shown in Figure 3 (accession number Y09062): Box A: CAAACCTCAACACCTCAACGGCCCTTATCAGGGCCACCA (spanning nucleotides 32 to 70); Box B: AGTCTCTGTAATTCATAATAGTCTCTGTAATTCATAAT (spanning nucleotides 90 to 127); DR: CAAACCTCAACACCTCAAC (spanning nucleotides 32 to 51); IR: CTCAACGGCCCTTATCAGGCCCACCAA (spanning nucleotides 45 to 71); (TC)14:CCTCTCTCTCTCTCTCT CTCTCTCTCTCTCTGTCT (spanning nucleotides 200 to 234). The GAGA oligonucleotide, GGGAGGGAGAGAGAGAGAGAGA, spans nucleotides −200 to −177 from the transcription starting site of the H2A promoter.

Microinjection and transgene expression

Plasmids were linearized with *Hin*dIII or *Cla*I restriction enzymes whose sites are located in the polylinker regions, 5′ and 3′ to the CAT sequence, respectively. Linearized plasmids were brought to a total concentration of 240 μg/ml with threefold excess of *Hin*dIII-digested *P. lividus* sperm DNA of roughly 5 kb. Unfertilized eggs from a mature female were dejellied in pH 5.0 sea water, immobilized on Petri dishes with 1% (w/v) protamine sulfate and injected into the cytoplasm with 1-2 pl DNA in 25 % glycerol solution. Microinjected eggs were fertilized and embryos raised up to the early gastrula stage (14 hours development). 3000 uninjected embryos at the same developmental stage were then added as carrier, and embryos collected by low speed centrifugation. Total RNA was extracted by one hour incubation at 55 °C in a solution containing 200 μg/ml Proteinase K and phenol extraction. Nucleic acid samples were digested with RNase-free DNase I, and RNA from 50 microinjected embryos was hybridized with [^{32}P]UTP-labelled antisense CAT or EGFP RNA transcribed *in vitro* from the H2A-spacer-CAT and GFP plasmids. Hybridisation conditions, RNase digestion and gel fractionation of the RNase-resistant hybrids were as previously described.[52]

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Tetracycline-Regulated Gene Expression Switch in *Xenopus laevis*

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***Xenopus* is a well-characterized model system for the investigation of biological processes at the molecular, cellular, and developmental level. The successful application of a rapid and reliable method for transgenic approaches in *Xenopus* has led to renewed interest in this system. We have explored the applicability of tetracycline-regulated gene expression, first described by Gossen and Bujard in 1992, to the *Xenopus* system. By optimizing conditions, tetracycline repressor induced expression of a luciferase reporter gene was readily and reproducibly achieved in both the *Xenopus* oocyte and developing embryo. This high level of expression was effectively abrogated by addition of low levels of tetracycline. The significance of this newly defined system for studies of chromatin dynamics and developmental processes is discussed.** © 2000 Academic Press

Key Words: **Xenopus; chromatin; transcription; development; gene expression; tetracycline.**

INTRODUCTION

The *Xenopus* oocyte and embryo have provided powerful model systems for the elucidation of mechanisms governing cellular and developmental processes [1–4]. In the case of the embryo, the description and use of a rapid and reliable *Xenopus* transgenic approach [5–8] have given a new perspective to *Xenopus* developmental studies [9]. This approach permits the overexpression of gene products in every cell of the organism or in a specific tissue. With this new technology comes the need to define parameters for its effective application, including the adaptation of existing transgenic methodologies. One of the invaluable features of the *Xenopus* oocyte system is its capacity to efficiently transcribe foreign genes encoded on microinjected plasmids following their assembly into chromatin [1, 10–13]. Recent evidence has suggested that processes regulating chromatin stability are linked to the transcriptional regulatory machinery (reviewed in [14]), highlighting the need to examine nuclear processes in a chromatin context. Since both transcription and replication can be assessed on templates assembled into chromatin using the *Xenopus* system, regulatory tools for use in the *Xenopus* oocyte or embryo would have important applications for the study of interactions between chromatin and the transcription and replication machinery. One such regulatory tool is the tetracycline-mediated gene expression switch.

Tetracycline-controlled gene expression was first described by Gossen and Bujard in 1992 and utilizes the very specific and high affinity binding of the *E. coli* tetracycline repressor protein (tetR) to its operator sequence (tetO) [15]. Using a fusion protein consisting of tetR fused to the VP16 activation domain (tTA) in HeLa cells, a luciferase reporter gene was activated up to five orders of magnitude and "turned off" to basal levels by the addition of low amounts of tetracycline to the tissue culture media. This tightly regulated genetic switch has been employed in a variety of studies where conditional gene expression is required. It has been used successfully in transgenic mice [16–18], where it is particularly appealing when the gene products under study are toxic or inhibitory to embryonic development. To address more diverse questions, the effective binding of tetR to its operon sequence has been exploited for purposes other than gene regulation. For example, it has been used in yeast to mark a specific region of DNA for mapping of sister chromatid separation with a tetR/GFP fusion protein bound to an array of tetO sites [19–21]. In the context of chromatin, tetR was demonstrated to form a physical boundary to nucleosome mobility in an *in vitro Drosophila* assembly system, thereby establishing a means to functionally analyze the chromatin remodeling machine CHRAC [22]. Here we describe, for the first time, the optimal conditions for successful application of this tetracycline-regulated switch for *in vivo* approaches in both the *Xenopus* oocyte and embryo.

MATERIALS AND METHODS

Antibodies and Constructs

pUHC13.3 contains seven tetO binding sites upstream of the minimal CMV promoter driving the luciferase reporter gene and pUHD15.1 expresses tTA protein. Both were generous gifts from S. Robine and have been previously described [15]. tTA mRNA was transcribed *in vitro* from the pSP65tTA plasmid constructed by in-

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serting the tTA coding region from pUHD15.1 into pSP65 by *Eco*R1/*Bam*H1 digestion and ligation. M13E4tetO was produced by removing the five Gal4 binding sites from M13E4G5 [2] by a *Hin*dIII/*Bam*HI digest and blunt ligation to the seven tandem repeats of tetO removed from pUHC13.3 by *Xho*I/*Stu*I digestion. The sequences and orientation of constructs were confirmed by sequencing. The TetR monoclonal antibody was raised against the tetracycline-responsive transcriptional activator tTA (Clontech catalog no. 8632-1) and was used at a 1:500 dilution for Western blotting according to standard protocols.

Xenopus Microinjection Strategy

Stage VI *Xenopus* oocytes were surgically removed and treated with collagenase as previously described ([11]; for methodological reviews, see [23]). *In vitro* transcribed tTA mRNA (quantity and quality assessed by UV analysis and electrophoresis) was injected into the cytoplasm of stage VI oocytes using a Drummond Nanoject automatic injector and incubated at 16°C overnight to allow tTA protein expression and accumulation. Approximately 18 h later, the tTA-regulated luciferase reporter pUHC13.3 [15] was injected into the nucleus and oocytes were incubated +/− 200 ng/ml tetracycline hydrochloride (Sigma) for a further 5 h to allow time for chromatin assembly on the reporter plasmid and luciferase expression. Ten healthy oocytes were recovered and lysed in 100 μl of lysis buffer, and the levels of luciferase reporter activity were assessed as described in the luciferase detection kit (Perkin–Elmer). Following lysis, DNA and RNA were analyzed as previously described [2].

Fertilized *Xenopus* eggs were coinjected into one blastomere at the 2-cell stage of development with 50 pg of pUHC13.3 reporter DNA and various amounts of tTA mRNA in a total volume of 26.7–32.2 nl as previously described [3]. Embryos were incubated +/− 2 μg/ml tetracycline hydrochloride for at least 14 h at 23°C. Alternatively, tetracycline was dissolved in water and coinjected into the embryos with pUHC13.3 and tTA mRNA to give a final concentration in the embryo of approximately 500 ng/ml. Embryos were lysed at various stages of development [24] and luciferase activity assessed.

Transcription Analysis

To assess the levels of either luciferase or E4 transcript accumulated per DNA template, a reverse transcription assay was performed as previously described [2]. Accumulation of luciferase mRNA transcript from 5 ng/oocyte pUHC13.3 luciferase reporter was detected by reverse transcription from an end-labeled oligo(5′-AGCCTTATGCAGTTGCTCTC-3′) annealed to luciferase mRNA extracted from the oocyte as previously described [2]. Extension gives rise to a product of 306 nucleotides. The same method was used to detect the E4 transcript, using in this case the end-labeled oligo(5′-CTTCACACCGGCAGCCTAACAGTCAGCC-3′), which produces a major product of 100 nucleotides. Efficiency of microinjection and chromatin assembly on transcribed templates was assessed by performing a supercoiling assay in parallel on DNA extracted from the same lysate used to prepare the RNA for reverse transcription analysis. Each lane of gel represents extension product from RNA accumulated in 2 oocytes. Quantification of transcripts and DNA recovery was performed with a PhosphoImager (STORM).

Supercoiling Assay

Following lysis of oocytes, a supercoiling assay was used as a measure of the efficiency of microinjection and assembly of injected templates into chromatin as previously described [2]. Each lane of the supercoiling assay represents DNA extracted from 5 oocytes from an injection of 5 ng/oocyte. Following electrophoresis, the DNA was Southern blotted and hybridized to M13E4tetO radioactively labeled with ^{32}P by random priming (Amersham Rediprime II RPN 1633).

RESULTS

Tetracycline-Regulated Gene Expression in the Xenopus Oocyte

It has been reported that the responsiveness of tetracycline-inducible systems can vary, depending on the cell type [25, 26]. In order to determine whether conditional gene expression could be achieved in *Xenopus* using the tetracycline-regulated system, we first asked whether expression of a luciferase reporter could be induced by tTA using a transient assay. The tTA protein was produced in the oocyte following cytoplasmic microinjection of *in vitro* transcribed tTA mRNA. This approach has previously been reported to be a reliable method for introducing foreign proteins into the oocyte [27–29], where the activity of injected RNA may be further improved by flanking the RNA with untranslated regions of the *Xenopus* globin gene. Therefore, we determined the activation threshold using 5 ng of microinjected reporter DNA when coinjecting increasing amounts of tTA mRNA. First, we confirmed that tTA protein was being produced in the oocyte at levels corresponding to the increase in injected mRNA (Fig. 1A). Second, we measured accumulation of luciferase mRNA by a reverse transcription assay (Fig. 1B). When the level of luciferase transcript was assessed, it was evident that an increase in activation of transcription from this reporter could be obtained using up to 75 ng of tTA mRNA, with 5 ng of reporter DNA reaching an optimal induction between 60 and 75 ng (Fig. 1B). The amount of tTA mRNA required to reach this optimal level is within the previously reported limit of 100 ng of mRNA that can be expressed by a single oocyte [30]. Factors available for luciferase protein expression also appear to be limiting since luciferase activity reaches a peak at 45 ng of injected tTA RNA (Fig. 1C), indicating that when high quantities of DNA are required, transcription efficiency should be assessed by analysis of the resulting transcript rather than the protein product.

Luciferase activity from the pUHC13.3 reporter was subsequently used as a measure of tTA-regulated expression of low levels of reporter since it has been well characterized for transient assays in tissue culture cells [15, 31, 32]. Figure 2A illustrates the experimental strategy taken for introduction of the tTA protein and luciferase reporter and analysis of products in the *Xenopus* oocyte. With this strategy, a range of amounts of both reporter plasmid and tTA mRNA gave high levels of reporter induction (Fig. 2B), achieving almost 50-fold induction of luciferase activity above basal levels expressed from the reporter without the tTA activator. An optimal induction was reached when 250 pg of reporter and 15–22 ng of tTA mRNA were microinjected since no significant increase in activation was

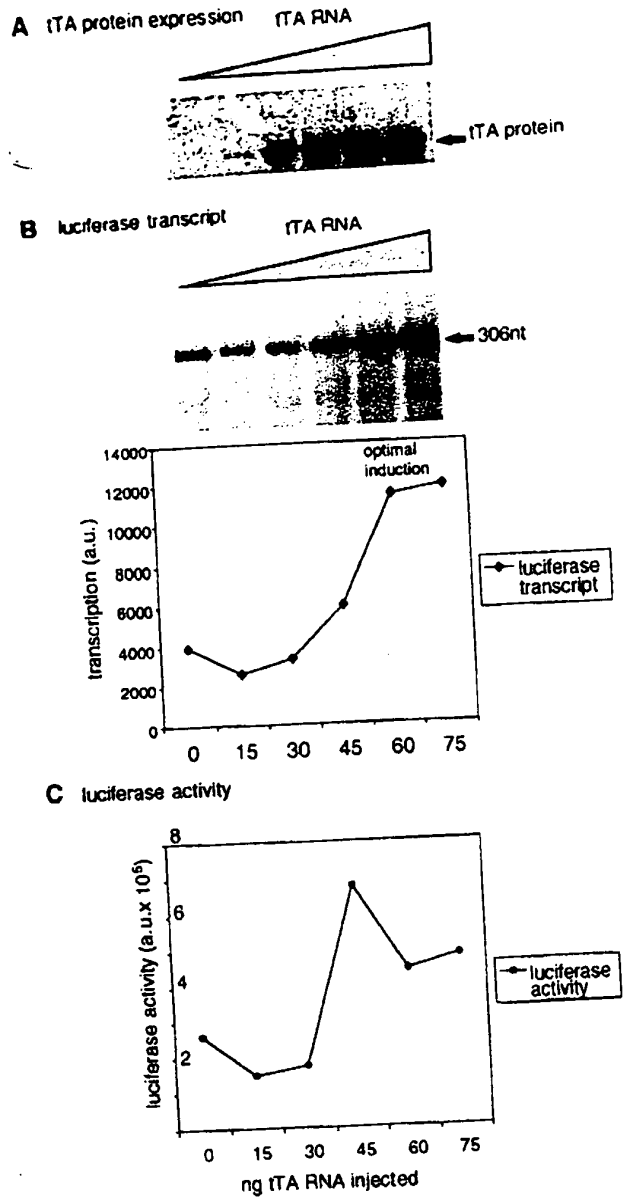

FIG. 1. Optimizing conditions for microinjection of tTA mRNA in the *Xenopus* oocyte. (A) Western analysis of *in vivo* translated tTA protein from oocyte extracts. Stage VI *Xenopus* oocytes were injected into the cytoplasm with the following increasing amounts of *in vitro* transcribed tTA mRNA: 0, 15, 30, 45, 60, and 75 ng/oocyte. Following injection, oocytes were incubated overnight for tTA protein expression and accumulation. Fifty oocytes were homogenized for each variable and centrifuged at 35,000 rpm and the clear protein extract layer was removed. Extract from the equivalent of 2 oocytes was then analyzed by Western blotting and revealed using a TetR monoclonal antibody (Clontech catalog no. 8632-1) at a 1:500 dilution and chemiluminescence according to manufacturer's instructions (Pierce SuperSignal, catalog no. 34080). (B) tTA-activated transcription from the pUHC13.3 reporter. (upper panel) Transcription of luciferase mRNA from 5 ng of pUHC13.3 luciferase reporter was detected by reverse transcription as described under Materials and Methods. The expected size of the reverse transcription product is given at 306 nt. A range of tTA mRNA of 0, 15, 30, 45, 60, and 75 ng/oocyte was injected in each case. (lower panel) Graphic illustration of accumulated luciferase mRNA, as described above, analyzed by phosphoimaging of the upper panel reverse transcription reaction. Optimal induction is indicated. (C) tTA-activated luciferase activity. Expression of luciferase from 5 ng of pUHC13.3 luciferase reporter was measured by a luciferase assay as described under Materials and Methods. A range of amounts of tTA mRNA of 0, 15, 30, 45, 60, and 75 ng/oocyte was injected as indicated.

detected despite the increased tTA RNA injected. This suggests that at this level all tTA binding sites are occupied. Significantly, addition of 200 ng/ml of tetracycline to the culture media was sufficient to completely abrogate this induction (Fig. 2B). Therefore, low levels of injected reporter DNA produced conditional gene expression, illustrating the effectiveness of this regulatory system in the *Xenopus* oocyte.

Tetracycline-Regulated Gene Expression during Development

Because of the high specificity and the low toxicity of the tetR protein and the tetracycline effector [15, 16], we wanted to determine the effectiveness of this system for use in the developing *Xenopus* embryo. Using an experimental strategy depicted in Fig. 3A, we coinjected fertilized eggs with luciferase reporter and various amounts of tTA mRNA to define the optimal parameters for tTA-driven promoter activation. Levels of injected DNA were kept to a minimum (50 pg/embryo) since higher amounts of DNA injected during early development result in low survival rates [33]. The most effective level of tTA mRNA to coinject with 50 pg of reporter was assessed at two different stages of development and determined to be 15–22 ng/embryo (data not shown). With these optimal conditions, high and reproducible levels of conditional gene expression were achieved in the developing embryo (Fig. 3B) following the midblastula transition when zygotic transcription is initiated (reviewed in [34]). At stage 11, although there is background luciferase activity expressed from the reporter plasmid, indicating that the embryos have developed beyond the MBT, there is no evidence of induction of luciferase expression by tTA, suggesting that not enough time has elapsed to establish levels of tTA protein necessary for activation of transcription. By stage 12, however, there is an effective induction of luciferase expression by tTA to over 100-fold, which is maintained through early development, with the peak of activation at stage 19 producing over 200-fold stimulation above levels with the reporter alone. By stage 27, the level of activation has dropped to 74-fold, indicating that the maximal limits of the system may have been reached by this stage. Significantly, levels of injected DNA are reported to decline following gastrulation [35], which may reflect the inability of even high levels of injected

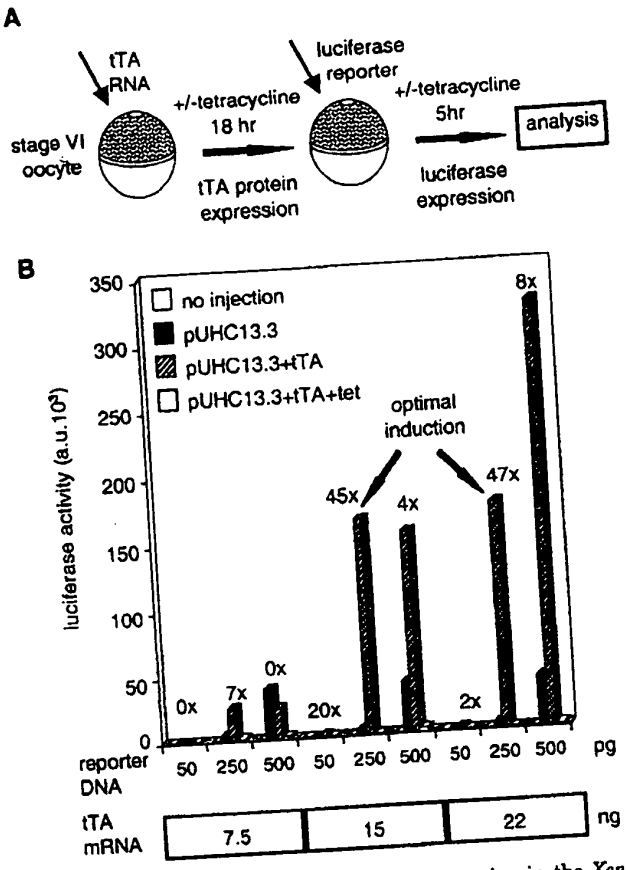

FIG. 2. Tetracycline-regulated gene expression in the *Xenopus* oocyte. (A) Experimental strategy for the tetracycline-regulated gene expression system in the *Xenopus* oocyte. Stage VI *Xenopus* oocytes were surgically removed and treated with collagenase as previously described [11, 23]. *In vitro* transcribed tTA mRNA was injected into the cytoplasm of oocytes, which were subsequently incubated at 16°C overnight +/− 200 ng/ml tetracycline hydrochloride (Sigma) to allow tTA protein expression and accumulation. Approximately 18 h later, the tTA-regulated luciferase reporter pUHC13.3 [15] was injected into the nucleus of the same oocytes and incubation continued +/− tetracycline for a further 5 h to allow time for chromatin assembly on the reporter plasmid and luciferase expression. (B) tTA induction of luciferase activity in *Xenopus* oocytes. Histograms illustrate the levels of luciferase activity, in relative light units, detected in the equivalent of 2 oocytes from the lysis of a total of 10 healthy oocytes per variable. Levels of induction and its abrogation on addition of 200 ng/ml tetracycline are shown after injecting 50, 250, or 500 pg of pUHC13.3 luciferase reporter and either 7.5, 15, or 22 ng of tTA mRNA/oocyte as indicated. Optimal induction and fold activation +tTA are indicated.

tTA mRNA to increase luciferase expression at this time. The above results indicate that the optimal induction of gene expression driven by the tTA protein using this transient assay is obtained at stage 19 of development, with high conditional activation achieved from stages 12 through to at least 27 using a ratio of 1:440 coinjected reporter DNA to tTA mRNA. We confirmed that the injected mRNA was translated into tTA protein by Western blotting of embryo extracts and that this level of mRNA was not inhibitory to early embryonic development (Fig. 3C). Injected DNA has been shown to persist for many months at low levels in the developing *Xenopus* [33, 35] probably following its integration into the host cell genome, suggesting that a mosaic pattern of induction may be achievable even at advanced stages of development using the protocol outlined in Fig. 3A. In fact, we were able to detect some activity from the luciferase reporter to the swimming larvae stage (stage 43) (data not shown).

Although it is clear that tTA can reach its tetO binding site to activate transcription in the embryo, results from these experiments indicate that tetracycline in the culture media has no effect on this activation (Fig. 3B, gray histograms). In a subsequent tetracycline titration experiment, a similar lack of effect was observed using 200 ng/ml and 0.02 mg/ml tetracycline (data not shown). Very high levels of tetracycline caused an arrest in development (at stage 13 for 0.2 mg/ml and stage 7 in the case of 2 mg/ml). Although the tetracycline-mediated shutoff of gene expression by tTA can be effectively achieved in tissue culture cells [15, 30] and in the *Xenopus* oocyte (Fig. 2B), it is not as rapidly established in whole organisms. Tetracycline can reduce levels of tTA-induced expression in transgenic mice implanted with slow-release tetracycline pellets, but this is often measured following 7 days of implantation [16] and variations in effectiveness with tissue type have been shown to exist [16–18]. We reasoned that *Xenopus* embryos could be more resistant than oocytes to the diffusion of tetracycline from the culture media. To address this question, we coinjected tetracycline with tTA mRNA and reporter DNA rather than adding tetracycline to the culture media. With this protocol, tTA-induced activation of luciferase was abolished by tetracycline even though injection of tetracycline with the reporter alone did not change the basal levels of luciferase expression (Fig. 4). The elimination of luciferase activity by tetracycline injection (compare pUHC13.3 + tTA histogram to pUHC13.3 + tTA + tet histogram) is remarkable considering the lack of tetracycline-mediated shutoff detected in Fig. 3B at a similar stage of development (stage 12, gray histogram). The tetracycline-regulated release of tetR from its recognition site can thereby be achieved using this coinjection strategy; however, optimization of this parameter will be needed to fully exploit the tetracycline-regulated system in the embryo.

Chromatin Assembly Coupled to Second-Strand Synthesis Eliminates Background Expression

To regulate gene expression in either the oocyte or as an integrated gene in a transgenic frog, the tTA protein must be competent to access its binding site on a DNA

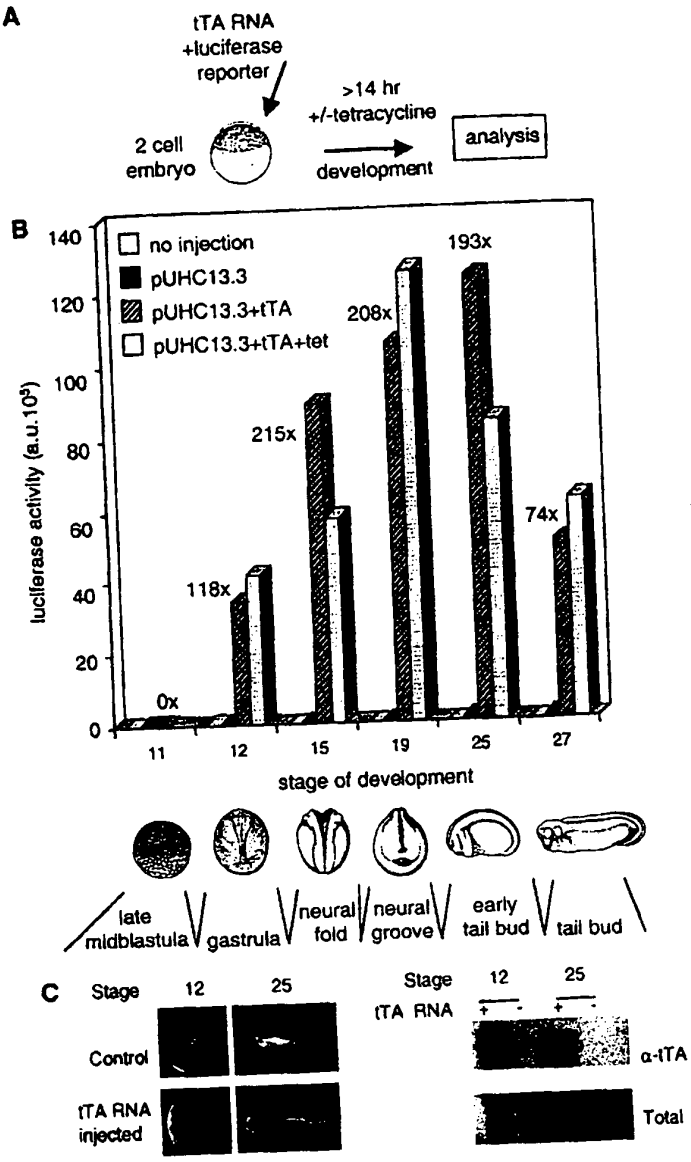

FIG. 3. Tetracycline-regulated gene expression during *Xenopus* development. (A) Experimental strategy in developing embryos. Fertilized *Xenopus* eggs were coinjected into one blastomere at the 2-cell stage of development with 50 pg of pUHC13.3 reporter DNA and various amounts of tTA mRNA in a total volume of 26.7–32.2 nl as previously described [3]. Embryos were incubated +/− tetracycline hydrochloride for at least 14 h at 23°C and lysed at various stages of development [24] to assess luciferase activity. (B) tTA induction of luciferase expression in the developing embryo. Histograms illustrate luciferase activity, in relative light units, detected from 2 embryos at various stages of development [24] as defined beneath (diagrams from Xenopus Molecular Marker Resource at http://vize222.zo.utexas.edu/). In each case, 22 ng of tTA RNA and 50 pg of pUHC13.3/embryo were coinjected and embryos incubated +/− 2 μg/ml tetracycline. Fold of activation +tTA is indicated for each developmental stage. (C) Expression of the tTA in the developing embryo. Left: pictures of control (top) and injected embryos with 25 ng of tTA RNA (bottom) were taken at the stages 12 and 25 according to [24]. Right: Western blot analysis of the corresponding embryos. The tTA protein is detected using the TetR monoclonal antibody (top panel) and the total protein is detected using Ponceau staining (bottom panel). The equivalent of 2 embryos is loaded in each lane.

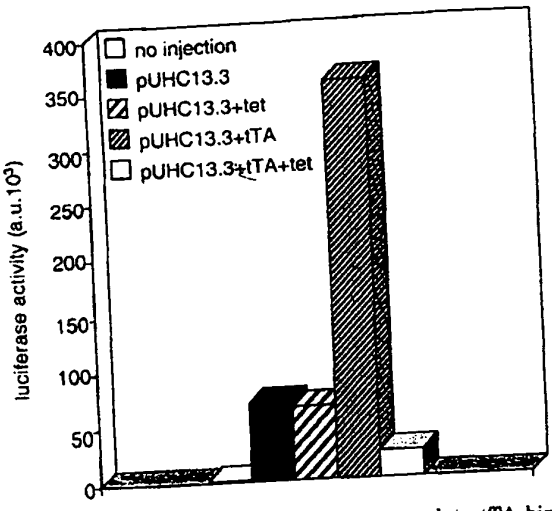

FIG. 4. Microinjection of tetracycline to regulate tTA binding. Histograms illustrate luciferase activity, in relative light units, detected from 3 embryos at stage 11–12 (midblastula) of development. In each case, 50 pg of pUHC13.3 and/or 22 ng of tTA mRNA per embryo was coinjected. Some samples, as indicated, were coinjected with a tetracycline solution to give a final concentration in the embryo of approximately 500 ng/ml.

template assembled into chromatin. Therefore, to extend the use of the induction system for analysis of microinjected reporter DNA assembled into chromatin, we further refined the conditions. Higher amounts of reporter DNA are required in this case since 1–5 ng of injected double-stranded plasmid DNA is the minimum amount necessary for effective chromatin assembly in the *Xenopus* oocyte [36]. We know from our initial experiments (Fig. 1) that 5 ng of reporter DNA is optimally activated by injection of 60–75 ng of tTA mRNA, indicating that tTA-regulated gene expression occurs on chromatinized templates, illustrating its potential as a tool either for regulation of integrated genes in transgenics or for *in vivo* chromatin studies. However, when we increase the amount of reporter DNA, background expression of luciferase is detected concomitant with a decrease in the activation potential (see Fig. 2B, 500-pg level, and Fig. 1B, basal transcription level). The high level of basal transcription from the minimal CMV promoter in this reporter construct is not surprising since genes driven by the CMV promoter are known to be highly expressed in the oocyte [2]. To eliminate this background expression from the reporter and to assess the effectiveness of this system in an integrated gene, we initiated a single-stranded (ss) template strategy. It has been shown that ss tem-

plates injected into oocytes are assembled into chromatin during the process of second-strand synthesis, which is repressive to basal transcription (initially reported in [2] and subsequently applied in [12, 29, 37, 38]). We reasoned that the introduction of the tTA-driven promoter on a ss template would provide a much more tightly regulated on/off system by repressing the effects of basal transcription. In addition, the ss template, once assembled into chromatin in the oocyte, would produce a template with the characteristics of an integrated promoter. Therefore, we constructed an M13 derivative (M13E4tetO) by removing the seven tetO binding sites from pUHC13.3 and inserting them into M13E4G5 [2] in place of the five Gal4 binding sites. Using this construct, we can compare both basal and tTA-activated transcription of the E4 gene during second-strand synthesis coupled chromatin assembly using a reverse transcription assay (Fig. 5A). When the ss template is injected, basal transcription is not detected (lane 1) whereas the double-stranded (ds) template shows background expression (lane 2) as expected. Although both the ss and ds templates were assembled into chromatin (as indicated by supercoiling, Fig. 5B), the tTA protein was able to overcome this chromatin-repressed state and activate transcription (lanes 3 and 7 for ss template and lanes 4 and 8 for ds template). Addition of 200 ng/ml of tetracycline to the oocyte culture media turned off the expression of the E4 gene (lanes 5 and 9 for ss template and lanes 6 and 10 for ds template). Therefore this ss template strategy provides a tightly regulated system to examine questions related to transcription from chromatin templates in the oocyte. It also indicates that the tetO binding sites would be accessible to tTA protein in an integrated promoter and that basal expression would be eliminated, providing an attractive mechanism for regulating overexpression of otherwise deleterious gene products in a transgenic *Xenopus*.

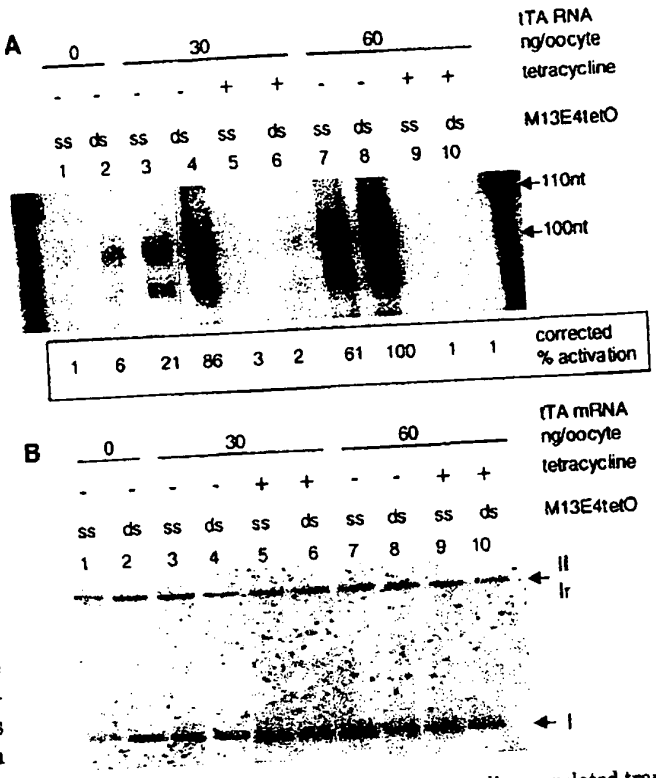

FIG. 5. Single-stranded strategy for tetracycline-regulated transcription. (A) Tetracycline-regulated E4 transcription. Transcription of the E4 gene from microinjected ss or ds M13E4tetO in *Xenopus* oocytes was detected using the reverse transcription assay described under Materials and Methods from oocyte extracts using the strategy illustrated in Fig. 2A. Either 5 ng/oocyte M13E4tetO alone (lanes 1 and 2) or in combination with 30 ng of tTA mRNA (lanes 3–6) or 60 ng of tTA mRNA (lanes 7–10) were microinjected into oocytes. Tetracycline (200 ng/ml) was added to the culture media in some cases (lanes 5, 6, 9, and 10). For each variable, 15 oocytes were lysed and the equivalent of 10 oocytes used for RNA extraction (A) and 5 oocytes for DNA extraction and supercoiling assay (B). Each lane represents mRNA extracted from 2 oocytes. The percentage activation is given for each lane corrected for amount of supercoiled template as shown in B. Marker at 110 nt is shown on right and left of gel. (B) Chromatin assembly on microinjected M13E4tetO templates. The supercoiling assay was used as a measure of chromatin assembly in the oocyte. Fifteen oocytes from each variable were pooled and lysed, with the equivalent of 10 oocytes used for RNA extraction as described above and 5 oocytes for DNA extraction and supercoiling assay. Lanes are the same as described for A. In each case, the efficiency of the microinjection was determined based on the amount of circular supercoiled DNA (I). The different forms of DNA are indicated as circular supercoiled (I), relaxed (Ir), and nicked (II).

DISCUSSION

In this report we define the optimal conditions for tetracycline-regulated gene expression in the *Xenopus* system. Information presented in Fig. 1A clearly shows that the tTA activator can be expressed to high levels in the oocyte. Importantly, the ability to assess the introduction of the tTA protein at the single-cell level in the *Xenopus* oocyte enabled us to determine that high levels of tTA (expressed from 75 ng of mRNA; Fig. 1A) can be introduced into an oocyte with no detectable deleterious effects on transcription (Fig. 1B). High levels of tTA protein can also be expressed in *Xenopus* embryos without gross developmental abnormalities (Fig. 3C). This is of interest since the tTA protein has been suggested to have toxic effects [17]. We also know that this tTA protein binds to the tetO sites since it activates luciferase expression 50-fold in the oocyte and over 200-fold in the developing embryo. The advantage of the tetR system over other activating systems such as hormone/receptor-driven systems [12, 29, 39] is the capacity to turn off activation, permitting gene activation or repression over a defined window, making it ideal for developmental applications and transgenics.

Refinements in restriction-mediated transgenic sys-

tems for *Xenopus* [5] allow overexpression of a particular gene product such as a kinase-deficient dominant-negative FGF receptor [6]. Application of tetracycline-regulated expression would improve its regulatory potential. During early stages of development, gene expression could be controlled by tetracycline injection. At later stages, when tadpoles or adults are feeding, tetracycline could be added to the food or water supply. In tetracycline-regulated mouse transgenics, a reverse rtTA induction system [18], where addition of tetracycline results in induction rather than shutoff, has overcome problems associated with the tTA system, where the half-life and clearance of the inducer are required for regulation (reviewed in [40]). This reverse system may prove to be a more effective alternative in the *Xenopus* embryo to maintain a prolonged shutoff of gene expression followed by a rapid burst of activation during a specific developmental stage or in a specific tissue. This burst of activation could be readily monitored by coupling the expression of the gene of interest to a fluorescent marker protein such as GFP.

Fluorescent markers have been used to track cellular processes by linking them to proteins and following their path during cell division or development. In such an approach, the LacI repressor fused to GFP was used to follow the localization of lactose operon operator sequences inserted into replication origin regions in *B. subtilis* [41]. An elegant series of experiments in yeast [19–21] make use of the tetracycline operator/repressor system to follow sister chromatid separation to identify factors involved in sister chromatid cohesion. The efficiency of expression and binding of tetR to its operon in *Xenopus* demonstrated in this report would facilitate the rapid application of such approaches to the study of nuclear dynamics in this higher eukaryotic system.

We are presently applying the tetracycline-regulated system to explore questions related to chromatin dynamics. Recent reports examining the action of the chromatin-remodeling machines in modifying chromatin suggest a tracking or sliding mechanism rather than the previous notion of nucleosome displacement [22, 42, 43]. In this context the binding of tetR has been used as a physical boundary to the mobility of nucleosomes during *in vitro Drosophila* chromatin assembly [22], lending further credibility to a sliding mechanism of action for CHRAC. We are using tetR as a barrier to chromatin assembly to plot the progress of bidirectional repair coupled chromatin assembly [44, 45] along DNA.

In conclusion, we have extended the application of tetracycline-regulated gene expression by defining the optimal parameters for its use in both the *Xenopus* oocyte and developing embryo. In both cases conditional expression of a gene of interest can be reliably and reproducibly achieved, adding to the already well-established usefulness of the *Xenopus* model system.

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Appendix E

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TECHNICAL ADVANCE

A chimeric transactivator allows tetracycline-responsive gene expression in whole plants

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Summary

The chimeric transcriptional activator tTA, a fusion between the Tn*10* encoded Tet repressor and the activation domain of the *Herpes simplex* virion protein VP16, was stably expressed in transgenic tobacco plants. It stimulates transcription of the β-glucuronidase (*gus*) gene from an artificial promoter consisting of 7 *tet* operators and a TATA-box. Tetracycline, which interferes with binding of tTA to operator DNA, reduces *gus* expression over several orders of magnitude. This stringency of regulation suggests that the system can be used to construct transgenic plants encoding a potentially lethal gene product. Furthermore, the specific and fast inactivation of tTA allows study of the stability of RNAs and proteins.

Introduction

Expression of foreign genes in transgenic plants is a widely used tool to confer new characters to different species (Schell, 1987; Willmitzer, 1988). In addition, enhancing or reducing the expression of endogenous genes helps us to understand the contribution of a defined gene product to the phenotype. The ability to control expression of a gene via a highly specific mechanism offers unique opportunities to study the physiological functions of certain gene products at different stages of development. The correlation of the phenotype with the kinetics of induction allows differentiation between primary and secondary consequences, which generates another advantage of a regulated expression system. Moreover, a stringently regulated promoter is absolutely required, if the expression of a gene product of interest interferes with the regeneration process.

Ideally, an inducible promoter should show extremely low or no basal levels of expression in the absence of inducing conditions, a high level of expression in the induced stage, and an induction scheme that does not otherwise alter the physiology of the plant. The last requirement, especially, renders the use of endogenous promoters, that respond to stimuli like heat (Schöffl *et al.*, 1989), wounding (Keil *et al.*, 1989), nitrate (Back *et al.*, 1991) or light (Gilmartin *et al.*, 1990) less favorable. A more promising approach is to combine regulatory control elements from other organisms, that respond to signals usually not encountered by a plant, with the general plant transcription machinery.

Based on this idea, two different concepts of gene control can be realized, i.e. promoter-repressing systems and promoter-activating systems. One way to construct a promoter repressing system is to use bacterial repressors to compete directly with plant transcription factors and/or RNA polymerases for binding (Gatz *et al.*, 1992; Wilde *et al.*, 1992). Using the Tn*10* encoded Tet repressor (TetR) in combination with a suitably engineered Cauliflower Mosaic Virus (CaMV) 35S promoter with three integrated *tet* operator sites we have succeeded in constructing a tightly repressible expression system (Gatz *et al.*, 1992). The DNA-binding affinity of TetR can be abolished by low amounts of tetracycline (Tc). The high equilibrium association constant of about 10^{-9}M for the repressor–inducer complex (Takahashi *et al.*, 1986) ensures efficient induction at Tc concentrations that do not even inhibit the growth of procaryotes (Geissendörfer and Hillen, 1990). Addition of Tc leads to a 200- to 500-fold induction of promoter activity throughout intact tobacco plants without any obvious inhibition of plant growth (Roeder *et al.*, 1994).

In this paper we describe the characterization of a Tc-dependent expression system in transgenic plants that combines the features of TetR with those of a promoter-activating system. Because of the modular organization of transcription factors (Frankel and Kim, 1991), eucaryotic activation domains can be fused to procaryotic repressor proteins thus turning them into transcriptional activators (Brent and Ptashne, 1985; Labow *et al.*, 1990). Fusion of

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TetR to the activation domain of virion protein 16 (VP16) of *herpes simplex* virus did not significantly alter the DNA-binding properties and the Tc inducibility of the TetR moiety (Gossen and Bujard, 1992). In Hela cells, this chimeric transcriptional activator (tTa) stimulated transcription from a minimal promoter sequence combined with *tet* operator sequences. Addition of Tc reduced gene expression over several orders of magnitude down to background levels.

Results

The Tc-controlled transcriptional activator tTA is functional in transiently transformed plant cells

To establish a gene expression system that can be negatively regulated by Tc we started with the construction of two types of DNA constructs termed 'activator plasmids' and 'target plasmids'. The 'activator plasmid' contains the chimeric *tTA* gene encoding amino acids 1–207 of TetR (Postle *et al.*, 1984) fused in frame to amino acids 363–490 of the transcriptional activator VP16 (Triezenberg *et al.*, 1988) under the control of the CaMV 35S promoter (Benfey *et al.*, 1990; Figures 1 and 2). The 'target plasmid' was made by replacing the enhancer sequences of the CaMV 35S promoter (sequences upstream from position −53) by a DNA fragment containing seven *tet* operator sites (Figures 1 and 2). The capacity of tTA to activate gene expression in plant cells was assessed by measuring Gus activity in extracts prepared from tobacco cells transfected with the 'target plasmid pUC-Top10' and increasing amounts of the 'activator plasmid pUC-TetVP16'. The left panel of Figure 1 contains five pieces of information;

(i) the VP16 activation domain is able to stimulate transcription in tobacco cells;

(ii) increasing the amount of 'activator plasmid' beyond an optimal level leads to a decrease in transcriptional activation;

(iii) when given optimal levels of 'activator plasmid', expression levels are twofold to threefold higher than those mediated by the CaMV 35S promoter;

(iv) in the absence of the 'activator plasmid', the 'target construct' mediates levels of gene expression which range between 10 and 18% of the maximal level obtained with optimal amounts of the 'activator plasmid';

(v) Tc reduces activation by tTA. When added at concentrations larger than 7 mg l^{-1} the residual Gus activity amounts to roughly the same level as mediated by the 'target plasmid' alone.

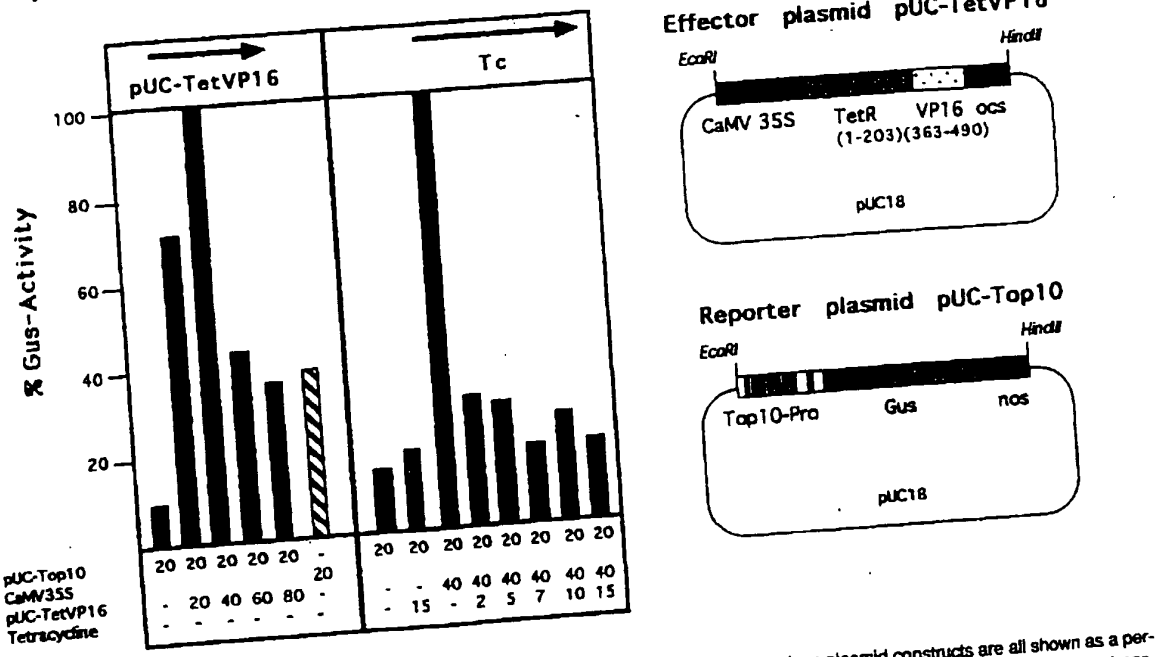
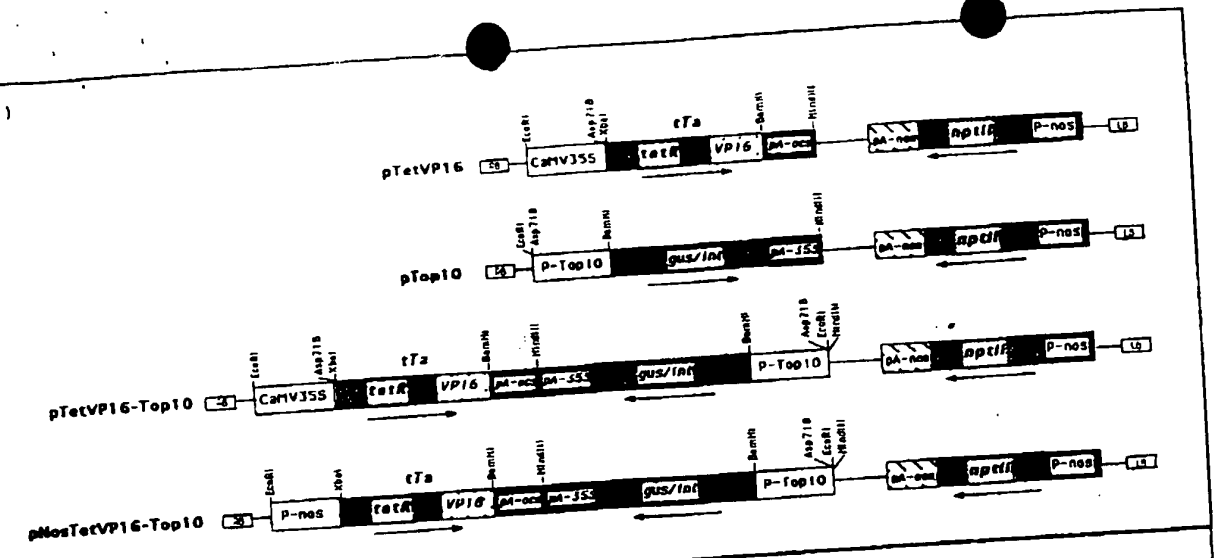


Figure 1. Transient analysis of tTa activity in tobacco protoplasts.
Left panel: relative Gus levels showing tTA function in plant protoplasts. The Gus levels detected with the various plasmid constructs are all shown as a percentage of the amount obtained when cells were co-transfected with 20 µg pUC-Top10 and 40 µg pUC-TetVP16. Below the graph the amount of the transfected plasmids is indicated in µg. pAT1 contains a CaMV 35S promoter derivative 5' to the *gus* gene (Gatz *et al.*, 1991, pAT1 is pAT2, except that pUC18 is the vector). Sheared salmon sperm DNA (carrier) was added such that plasmid and carrier DNA totalled 120 µg. The amount of Tc is given in mg l^{-1}. In the experiment showing the effect of Tc, six batches of protoplasts transfected with 20 µg pUC-Top10 and 40 µg pUC-TetVP16 were combined after PEG treatment and divided afterwards to allow cultivation with increasing amounts of Tc. Black columns; expression levels of the Top10 promoter; shaded column; expression level of the CaMV 35S promoter.
Right panel: maps of the 'activator plasmid pUC-TetVP16' and the 'target plasmid pUC-Top10'. For abbreviations see Figure 2.



(b)

EcoRI Sacl Asp718 Sacl *Operator #1*

GAATTC-GAGCTC-GGTACC-GAGCTC-GACTTTCACTTTTCTCTATCACTGATAGGGAGTGGTAAACTCGACTTTCATTT

Operator #2 *Operator #3*

TCTCTATCACTGATAGGGAGTGGTAAACTCGACTTTCACTTTTCTCTATCACTGATAGGGAGTGGTAAACTC

Operator #4 *Operator #5*

GACTTTCACTTTTCTCTATCACGGATAGGGAGTGGTAAACTCGACTTTCACTTTTCTCTATCACTGATAGGGA

Operator #6 *Operator#7*

GTGGTAAACTCGACTTTCACTTTTCTCTATCACTGATAGGGAGTGGTAAACTCGACTTTCACTTTTCTCTATCACTG

SnaBI TATA-BOX StuI XbaI XhoI

ATAGGGAGTGGTAAACTCGAGATAGAGTGATCTAGTCTTCGCAAGACCCTTTACGTATATAAGGCCTTTCTAGACATTTGCTCGAG

igure 2. Constructs.

a) Diagram of the chimeric genes used for the establishment of a Tc-responsive promoter in transgenic plants. pTetVp16 contains the transcriptional transctivator gene (*tTA*) under the control of the CaMV 35S promoter. pTop10 encodes the *gus/int* gene under the control of the chimeric promoter consisting of seven *tet* operator sites 5' to a TATA-box (TATATAA). pTetVP16-Top10 contains both chimeric genes on one T-DNA. pNosTetVP16-Top10 differs from pTetVP16 with regard to the promoter (P-Nos) driving *tTA* expression. RB, right border; LB, left border; pA, polyadenylation signal. P—promoter; *nptII*, neomycinphospho-transferase; *ocs*, octopine synthase; *nos*, nopaline synthase. *gus/int*: β-glucuronidase with an intron (Vancanneyt *et al.*, 1990). Arrows indicate the transcriptional orientation.

b) Sequence of the target promoter P-Top10. The sequences of the operators and the TATA-box are underlined, recognition sequences for restriction sites are written in italics.

Activity of the target promoter P-Top10 during the regeneration process

In order to characterize the function of tTA and its target promoter in stably transformed plants we cloned the respective chimeric genes on binary vectors which were designed for *Agrobacterium tumefaciens*-mediated gene transfer (Bevan, 1984). As a first step we replaced the *gus* gene of the reporter construct by a modified version that contains an intron (Vancanneyt *et al.*, 1990). Thus Gus activity of transformed plant tissue can be monitored very early after the co-cultivation step, because the gene is not properly expressed in *Agrobacterium*. Four different transgenic tobacco lines were generated. Figure 2 depicts the different chimeric genes that were cloned into BIN19. pTetVP16 encodes only the 'activator construct' whereas pTop10 carries only the 'target construct' adjacent to the chimeric kanamycin-resistance gene. pTetVP16-Top10 contains both constructs on the same T-DNA. To avoid a potential influence of the CaMV 35S enhancer on transcription from the target promoter P-Top10 the reporter construct was placed downstream of the chimeric *tTa* gene. A tail-to-tail orientation of both genes was chosen as a further precaution. pNosTetVP16-Top10 differs from pTetVP16-Top10 with regard to the promoter driving the expression of tTA: whereas pTetVP16-Top10 encodes

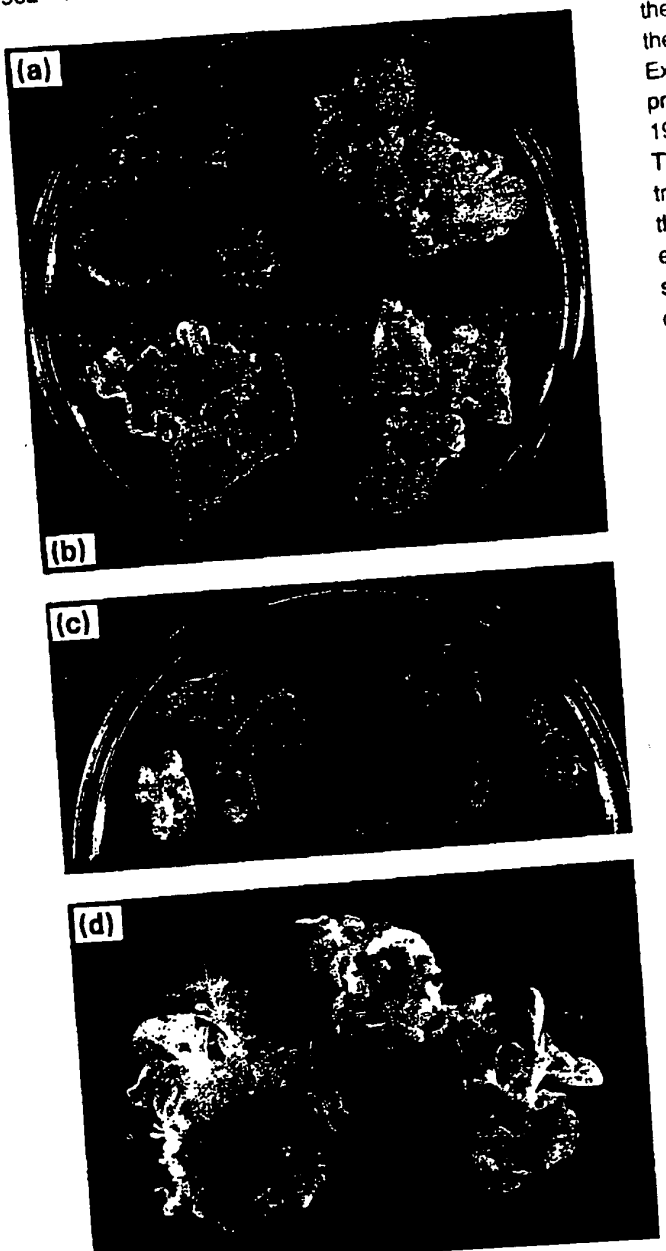


Figure 3. Gus expression in regenerating transformants.
(a) TetVP16-Top10, 36 days after transformation; (b) Top10, 36 days after transformation; (c) CaMV 35S/Gus-Int, 20 days after transformation; (d) pNosTetVP16-Top 10, 74 days after transformation. The Tc concentration in the medium was 1 mg l^{-1}.

tTA under the control of the CaMV 35S promoter, the Nos promoter (An *et al.*, 1986) was used in pNosTetVP16-Top10.

As shown in Figure 3(a), Gus activity is only detectable in explants transformed with pTetVP16-Top10, when grown in the absence of Tc. No activity is seen in leaf discs incubated on Tc-containing medium. Explants from the transformation with pTop10, a plasmid that contains only the 'target construct', did not express the *gus* gene, neither in the presence nor in the absence of Tc (Figure 3b). Explants from a control transformation with the CaMV 35S promoter driving the *gus/int* gene (Vancanneyt *et al.*, 1990) proved the specificity of the Tc effect (Figure 3c). The staining of explants from the pNosTetVP16-TOP10 transformation (Figure 3d) revealed Gus activity only in the callus stadium, where the Nos promoter is active enough to direct sufficient levels of *tTA* expression. In shoots, however, no activation of the *gus* gene was observed.

Regulation of the target promoter P-Top10 in transgenic plants

Gus activities of 60 shoots from TetVP16-Top10 plants, 50 shoots from Top10 plants and 20 shoots from Nos-TetVP16-Top10 plants were determined. After 30 min the reaction of TetVP16-Top10-derived extracts was stopped.

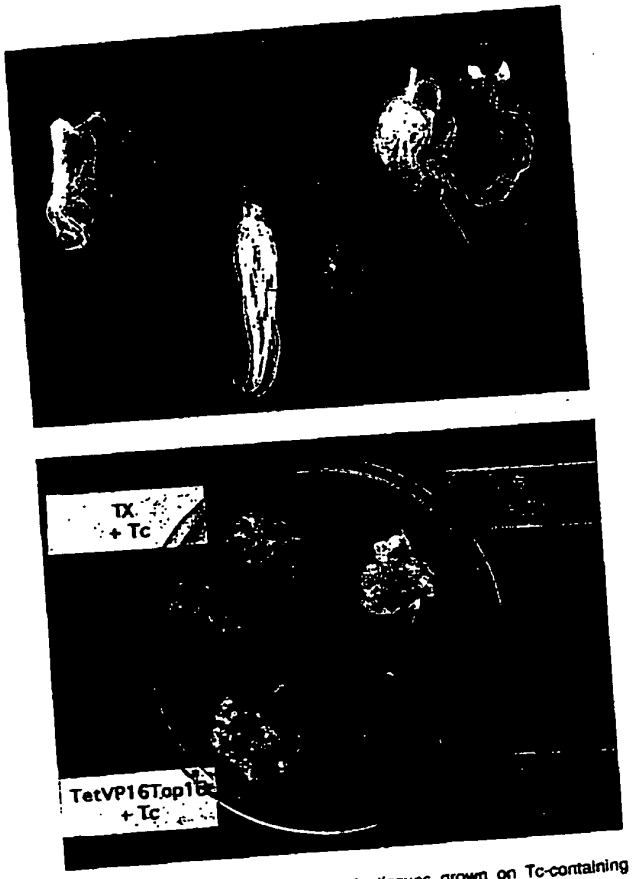


Figure 4. Inhibition of Gus activity in tissues grown on Tc-containing medium.
Upper panel: Gus expression in roots of plants nos 22, 24 and 30, grown either in the absence (–) or presence (+) of 1 mg l^{-1} Tc. Lower panel: transformed plants were re-regenerated either in the presence or absence of Tc. Plant TX contains the Tc-inducible Triple-Op promoter in front of the *gus* gene.

able 1. Gus activities of nine highest expressing TetVP16-op10 plants and a representative Triple-Op/gus plant (TX)

ransformant	pmol 4 MU min^{-1} mg^{-1} protein
	820
5	1550
6	3190
3	1780
2	1060
4	1470
0	2045
2	6070
3	7530
7	13 000
X	

Gus activity was determined using the fluorometric assay of efferson (Jefferson *et al.*, 1987). One leaf was taken when hoots had just formed small roots on kanamycin containing nedium.

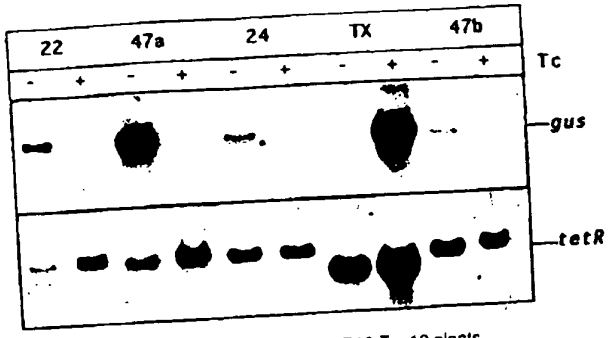


Figure 5. Northern blot analysis of TetVP16-Top10 plants.
RNA was prepared either directly from plants grown under axenic conditions on 2MS medium (lanes marked –) or after infiltration with 1 mg l^{-1} Tc and 48 h incubation on Tc-containing medium (lanes marked +). In lanes TX+ and TX– RNA of a transgenic plant containing the *gus* gene under the control of the Tc-inducible TX-promoter was loaded to demonstrate the reciprocal effect of Tc. The blot was first probed with a restriction fragment containing the *gus* coding region, and subsequently with a 695 bp tetR fragment.

The average Gus activity was calculated to be 660 pmol MU min^{-1} mg^{-1} protein (U). Expression levels of the nine highest expressing plants varied between 7530 and 820 U Table 1). These plants were kept for further analysis. Extracts from Top10 transformants and NosTetVP16-op10 transformants were incubated for 12 h in order to detect even low amounts of activity. Under these conditions only four Top10 plants showed a Gus activity of ca. 30 U. Activities in all the other shoots were indistinguishable from activities measured in untransformed control plants (10 U). To assess the effect of Tc on Gus activity of TetVP16-Top10 plants, we first compared tissues which were newly formed either in the presence or absence of Tc. Thus, we avoided potential complications due to the stability of the Gus protein. The upper panel of Figure 4 shows roots from plants nos 22, no. 24 and 30; the lower panel shows regenerating shoots from explants taken from plant no. 47 and from a transgenic plant expressing the *gus* gene under the control of the Triple-Op promoter (TX) which is negatively regulated by TetR (Gatz *et al.*, 1992). Even after staining overnight no detectable Gus signal was observed in roots grown in 2MS medium with 1 mg l^{-1} Tc indicating a very stringent regulation. Quantitation using the fluorometric assay (Jefferson *et al.*, 1987) revealed that tissue formed in the presence of Tc (roots, callus, regenerating shoots) never showed higher Gus activity than untransformed controls, even when the extracts were incubated for more than 12h. Under these conditions, the Triple-Op promoter, which is inducible by Tc, gives values between 100- and 250-fold regulation. As the Top10 promoter in the presence of Tc yields no activity above the background measured in untransformed control plants, we cannot calculate an equivalent numerical value for the efficiency of regulation. When shoots that had formed on Tc were placed on 2MS without Tc, Gus activity reaccumulated, which shows that the regulation is reversible.

The explants shown in the lower pannel of Figure 4 indicate that Gus expression levels are sensititive to Tc not only in newly formed shoots, but also in leaf explants, which had expressed Gus activity before being put on Tc. We confirmed this on the RNA level by Northern blot analysis of RNA from leaves being vacuum infiltrated with and without Tc (Figure 5). As expected, plants nos 22, 47 and 24 showed a Tc-mediated decrease in mRNA accumulation. Plant TX, which contains the *gus* gene under the control of the TetR-regulated promoter, shows the positive effect of Tc in that system. At the level of total RNA no background activity could be detected, with longer exposures showing only signals originating from cross-hybridization to ribosomal RNA. Whereas no. 47 showed the expected expression levels comparable with the levels given by the CaMV 35S promoter (lane 47a, see Table 1), plants nos 22 and 24 showed unexpectedly low mRNA levels. As the RNA was isolated 3 months after quantitation of Gus expression levels, it seems that the target promoter gets silenced when the plants grow older. This was confirmed by analyzing RNA of plant no. 47 1 year after the transformation. The decrease in the amount of expression is demonstrated in lane 47b (Figure 5). This instability of the activity of the target promoter was observed in all our transformants. Rehybridization of the Northern blot with the *tetR* probe revealed, that the silencing of the promoter was not due to reduced expression of *tTA* mRNA. Gus activities of 10 kanamycin-resistant seedlings from plants nos 13, 30 and 47 were determined to be 430 U, 50 U and 250 U, respectively, which still is significantly lower when compared with the expression levels of the young transformants (Table 1). All of them showed again stringent sensitivity to Tc.

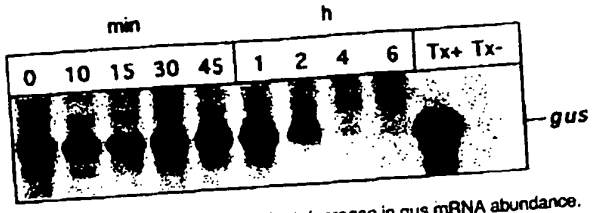


Figure 6. Kinetics of Tc-dependent decrease in gus mRNA abundance. Leaves form plant TetVP16-Top10#13 were infiltrated with 1 mg l^{-1} Tc in 50 mM sodium citrate and incubated on MS medium. RNA was extracted after the time points indicated above the lanes. In lanes TX+ and TX– RNA from a transgenic plant containing the *gus* gene under the control of the Tc-inducible TX-promoter was loaded. The blot was probed with a restriction fragment containing the *gus* coding region.

Time course of Tc-dependent Gus expression

The time course of Tc action on the steady-state levels of mRNA was analyzed in leaves of plant no. 13 which were treated with 1 mg l^{-1} Tc by vacuum infiltration. RNA was extracted after 10 min, 15 min, 30 min, 45 min, 1 h, 2 h, 4 h and 6 h. After a lag time of 1 h, the RNA decayed with a half life time of less than 1 h (Figure 6).

The time course of Tc action on the steady-state levels of Gus protein was analyzed at the whole plant level. Two cuttings of plant no. 22 were transferred into hydroponic culture in the greenhouse. When the plants had reached a height of 40 cm (10 leaves), Tc was added to one cutting. At regular intervals (every third or fourth day) samples were taken from the eight upper leaves of each plant and their Gus activities were determined. Values of the untreated plant were used as the 100% reference point each day. The mean value for each series of measurements was plotted against time on a half logarithmical scale (Figure 7). After 4 weeks, Gus activity had dropped down to 1% of the initial activity, but did not decrease further. In the experiment shown here, the half life of the Gus protein in green tobacco plants is ca. 3–4 days. However, the half life depended on the size of the plant. When plants with only four leaves were treated with Tc, Gus activity had a half life of approximately 1 day (data not shown).

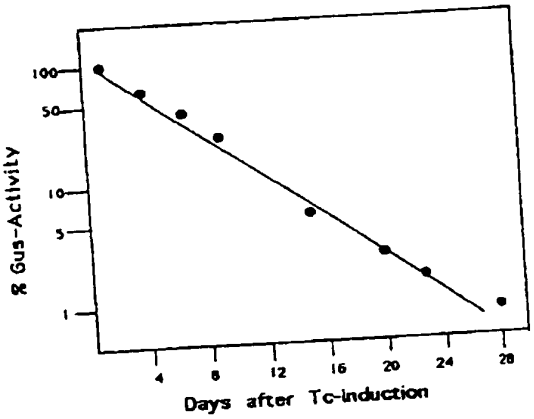


Figure 7. Kinetics of Tc-dependent decrease of Gus enzyme activity. Two cuttings of TetVP16-Top10#22 were grown in hydroponic culture in the greenhouse. 3, 7, 9, 15, 20, 23 and 28 days after the onset of Tc treatment of one plant; samples of eight leaves of each cutting were taken. The graph shows the mean values. Each day samples of the untreated control were calculated to be 100%.

Characterization of tTA expression in transgenic plants

Transgenic TetVP16 and TetVP16-Top10 plants were analyzed for the expression of tTA. Extracts from 20 TetVP16 plants were subjected to gel shift analysis and eight plants showing strong signals were chosen for RNA analysis (Figure 8a). *Tetvp16* RNA (*tTA* RNA) is clearly detectable, though less abundant than *tetR* mRNA, which was isolated from a transgenic plant transformed with pTet1 (Gatz *et al.*, 1991). pTetVP16 and pTet1 are identical, except for the 381bp extension of the *tetR* coding region. To correct for the amount of RNA loaded we rehybridized the blot with the *S4* probe, which encodes a ribosomal protein (Devi *et al.*, 1989). Even though slightly more RNA was loaded for plant TetVP16#17, it appears to encode the highest amount of tTA and may be used in the future for subsequent transformations with chimeric genes under the control of the Top10 promoter. tTA expression

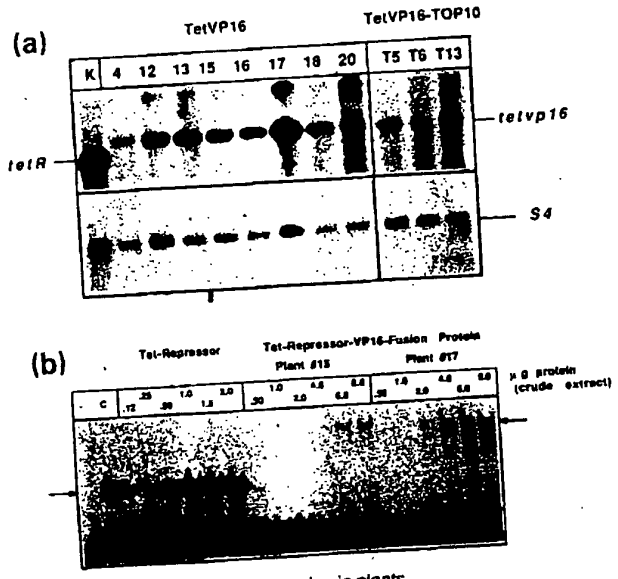


Figure 8. Analysis of tTa expression in plants.
(a) Northern blot analysis of TetVP16 and TetVP16-Top10 tranformants. Lane K contains RNA from the Tet1#2 transformant expressing high amounts of TetR (Gatz *et al.*, 1991). The blot was first probed with a restriction fragment containing the *gus* coding region and subsequently with the probe for the ribosomal protein S4.
(b) Gel shift analysis to compare TetR and tTA abundance in crude extracts from transgenic plants. End-labeled operator fragment (6 fmol) was incubated with increasing amounts of protein extract. Numbers above the lanes indicate the amount of protein in µg. In lane C TetR purified from *E.coli* extracts was loaded. Arrows point to the protein-DNA complexes.

levels of three plants transformed with pTetVP16-Top10, which showed different Gus expression levels (Table 1) were in the same range. The gel shift analysis provides biochemical evidence for tTA expression in transgenic plants (Figure 8b). Owing to the change in molecular weight and charge the complex has a reduced electrophoretic mobility as compared with the Tet repressor–operator complex.

Discussion

We have established a regulated promoter for transgenic plants whose activity is turned off in the presence of low amounts of Tc. Regulation is based on an activation mechanism: a chimeric protein (tTA) consisting of the Tn*10*-encoded Tet repressor (TetR) fused to the activation domain of a eucaryotic transcriptional activator (VP16) (Gossen and Bujard, 1992) was expressed in plants. When bound to an array of *tet* operator sequences upstream of the TATA-box of the CaMV 35S promoter it activates transcription *in vivo*. In the presence of Tc, which prevents tTA from binding to the operator sequences, the promoter is inactive. In contrast, the principle of our previously established Tc inducible system (Gatz *et al.*, 1992) is based on repression. The Tn*10*-encoded Tet repressor sterically interferes with the establishment of a functional transcription initiation complex if three operator sites are suitably engineered in the vicinity of the TATA-box of the CaMV 35S promoter. Addition of Tc induces gene expression because it prevents TetR from binding. For both expression systems the features of the Tn*10*-encoded TetR—namely its capacity to bind to operator DNA and the action of Tc to interfere with binding (Hillen *et al.*, 1984)—were exploited. This discussion includes a comparison of the promoter-activating versus the promoter-repressing system for the transcriptional regulation in plants.

Transient assays

Transient assays proved that tTA is functional in plants. This was expected in view of the observation that the acid domain of VP16, originally encoded by *herpes simplex* virus, can activate transcription in a variety of organisms like yeast (Berger *et al.*, 1992), mammalian cells (Sadowski *et al.*, 1988), insects (Wampler and Kadonaga, 1992) and plants (McCarty *et al.*, 1991). As in the other systems, we observe 'squelching' effects at higher tTA concentrations, which indicates that tTA interacts with some component of the transcriptional machinery, even if it is not bound to operator DNA (Berger *et al.*, 1990). At optimal concentrations, the activity of the tTA-driven promoter is higher than the activity mediated by the CaMV 35S promoter. However, its background activity in the absence of the activator was rather high (10–18% of the optimal tTA-dependent activity). It might well be that the sequence between the operators upstream of the TATA-box contains a cryptic activation site that is recognized in protoplasts. We have shown previously that the operator sequence itself does not mediate activation (Frohberg *et al.*, 1991). In addition, sequences between +1 and –50 in the same vector do not confer promoter activity (data not shown). Upon addition of Tc activity of the reporter construct dropped, similar to results obtained earlier in stably and transiently transformed Hela cells (Gossen and Bujard, 1992). However, at 2 mg l^{-1} Tc, a concentration which causes maximal induction in the repressed system, tTA is not completely inactivated. These results are in contrast to comparable transient expression experiments in mammalian cells, where even lower Tc concentrations completely inhibit tTA-dependent activation (Gossen and Bujard, unpublished results). As in stably transformed plants even 1 mg l^{-1} Tc is sufficient for complete inactivation of tTA (see below), we would assume, that the incomplete inactivation is not due to a reduced affinity of tTA for Tc. We would rather favor the explanation that at the high concentrations of transactivator and reporter DNA present in a transient expression system, the tTA–Tc complex might still cause some activation due to unspecific binding to the introduced target plasmid. In conclusion, these results indicate that the tTA- based system is not appropriate for the regulated expression in transient systems. In contrast, we do not find any background activity and maximal inducibility by Tc in transient assays when TetR is used to to repress transcription (Gatz *et al.*, 1992).

Stable transformants

The problems encountered in transient assays were not observed in stably transformed plants. Forty-six transgenic plants containing the Top10 construct did not show any detectable Gus activity, four plants showed low activity, probably due to the integration of the T-DNA in the proximity of enhancer elements that might interact with the TATA-element of the target promoter. If the chimeric *tTA* gene was introduced along with the target promoter, 46 out of 60 kanamycin-resistant plantlets expressed the *gus* gene if grown in the absence of Tc. The difference in the expression levels between the inactive state and the active state of this system is difficult to quantify because Gus activity in the presence of Tc is close to zero. Similar results were obtained in HeLa cells, where luciferase activity was less than 2 U in cells grown in the presence of Tc and up to 257 100 U in the absence of Tc resulting in a regulation factor of at least 1 × 105 (Gossen and Bujard, 1992). Gus activity in untransformed plants and TetVP16-Top10 plants grown in the presence of Tc was 10 U in our

hands, maximal activity was 7000 U. We therefore conclude, that the amount of regulation is greater than 700-fold, but it is very likely that this is an underestimation. In contrast, the regulation of the repressor based system does not exceed 500-fold under optimum conditions. This confirms the notion that promoter-activating systems are more efficient for the tight regulation of individual genes in higher eucaryotes than regulatory systems based on sterical interference. This may be partly due to the fact that transcriptional activators have free access to their target sites, whereas repressors compete with endogenous transcription factors for binding. Thus, higher levels of a repressor protein are needed for the same degree of occupancy of target sites. In addition, 50% occupancy of binding sites can be sufficient for transcriptional activation but not for stringent repression. In this regard, the copper-controlable gene expression system for whole plants, which uses a yeast metalloresponsive transcription factor to stimulate transcription in the presence of copper is—in theory—very promising (Mett *et al.*, 1993). However, the regulation was reported to be only 50-fold under optimal conditions, partly due to residual background activity. The steroid-inducible regulatory system, which is based on the glucocorticoid transcription factor, has been shown to be functional in transiently transformed protoplasts, but not in stably transformed tissue (Schena *et al.*, 1991). If even very low amounts of a gene product might be lethal for the plant, we would suggest use of the tTA-based system rather than the inducible promoter. Gene expression can be obtained if plants are removed from Tc-containing medium. However, gene activation depends on the dilution and/or inactivation of Tc *in planta*, a process that spans at least a week and may vary with growth and light conditions. Thus, a potential disadvantage of the system is, that gene expression cannot be obtained at a precise time point. For some applications, like the regulated excision of selective markers between *lox* sites (Dale and Ow, 1991), the exact length of the induction period would not matter: explants could be regenerated on a selective medium in the presence of Tc; upon cultivating without Tc after selection, the Top10 promoter driving the *lox* recombinase would be turned on in the whole plant.

When discussing efficiencies of regulatory expression systems the expression levels of the induced stage should be evaluated. Gus activities are usually indicated in pmol 4 MU min^{-1} mg^{-1} protein (Unit). However, data on the widely used CaMV 35 promoter activity, for instance, vary over a broad range. CaMV 35S promoter mediated Gus activities were reported to be 113 000 Units (average of 10 plants, Benfey *et al.*, 1989), 321 U (one selected plant, Jefferson *et al.*, 1987), 9000 U (average of 15 plants, Sanger *et al.*, 1990), 500 U (highest expressing plants, Comai *et al.*, 1990) and 130 000 U (one selected plant, Keil *et al.*, 1989). In our hands, the CaMV 35S promoter and its Triple-Op derivative yield activities between 10 000 and 30 000 U in representative plants. The activities mediated by the tTA-dependent Top10 promoter ranged between 1200 and 7000 U. Given the above-mentioned variations of the units determination we cannot relate that to the 1200 U reported for the copper-inducible system. Direct comparison of the tTA-based system with the CaMV 35S promoter, however, allows us to state that the activity is three to five times lower than the activity of the CaMV 35S promoter and its Tc inducible-derivative P-Triple-Op. This feature might be due to the closely linked location of the reporter construct and the activator construct. Because of the counterselection against high tTA expression (see Figure 8), only plants with T-DNA integrations in less highly expressed locations might have survived the regeneration process. It remains to be investigated if a second transfection of the TetVP16#17 plant with a Top10 construct yields higher activities. However, the initial activity was not stable. One year after the transformation event, plants kept in tissue culture consistently did not synthesize more than 400 U Gus. As shown by Northern blot analysis (Figure 6) this was not due to reduced transcription of the *tTA* gene. Also, in the progeny we could not detect the initial amounts of Gus activity. It remains to be investigated, whether methylation within this region or co-suppression due to multiple T-DNA insertions are responsible for the inheritable reduced stability.

Kinetics of the regulation

The possibility of switching off transcription from one specific promoter should allow analysis of mRNA or protein decay rates of individual genes. Thus the tTA-dependent promoter might provide a good alternative to the use of general inhibitors like actinomycin D and cycloheximide. When Tc was applied through vacuum infiltration into single leaves, gene expression of the inducible promoter reached maximal levels within less than 30 min, indicating a rapid inactivation of TetR (Gatz *et al.*, 1991). Tc-infiltrated leaves of a TetVP16-Top10 plant showed a response after a lag time of 1 h. From there on *gus* mRNA disappeared with a decay rate of less than 1 h. As decay rates depend on the age of the material, we would however suggest that, if the stabilities of two mRNAs are to be compared, both should be put under the control of the Top10 promoter and integrated simultaneously into one transgenic plant.

Application of Tc through the roots of a TetVP16-Top10 plant led to a decay of the protein following first order kinetics already 1 day after Tc treatment. The half life of the protein was 3–4 days in a plant 40 cm high. When younger plants were used, the protein disappeared three times faster. Again, we would suggest, that the system is

very useful for the direct comparison of the stabilities of two proteins.

Expression of tTA in transgenic plants

The transient assays already indicated, that high levels of *tTA* expression might lead to the inhibition of transcription due to squelching effects. Consistently we found that transgenic plants synthesize less mRNA for tTA than for TetR (Figure 8a). The gel shift analysis (Figure 8b) allows a rough estimation of the difference between TetR and tTA expression levels: 0.12 µg of the extract derived from plant Tet1#2 encoding TetR retard about the same amount of operator encoding DNA as 6 µg of the TetVp16#17 extract, indicating that tobacco plants tolerate about 50-fold less tTA than TetR. However, this estimation is based on the assumption that tTA binds operator DNA with a similar affinity as TetR, and that it is as stable in the crude extract as TetR. The Nos promoter, which is reported to be 30-fold less active than the CaMV 35S promoter (Sanders *et al.*, 1987), is too weak to synthesize sufficient amounts of tTA in leaves. The blue staining we observed in callus of NosTetVP16-Top10 plants proves that the construct is functional. We have found no direct comparison of Nos promoter activity in leaves and callus in the literature. However, it has been described, that the Nos promoter is under developmental control (An *et al.*, 1988) as well as wound inducible and auxin inducible (An *et al.*, 1990). Therefore it is very likely, that its expression is higher in callus than in leaves. This indicates that tTA levels have to exceed a certain threshold to be able to drive expression from the target promoter.

In conclusion, we have established a Tc-dependent expression system in transgenic plants and demonstrated its potential to regulate efficiently expression of a transgene. For some applications, this system, which is negatively regulated by Tc, might be superior to the previously established Tc-inducible promoter (Gatz *et al.*, 1992): (i) it is particularly useful if the stringency of the regulation has to be very high; (ii) It can be used for the study of mRNA and protein decay rates.

Experimental procedures

Plants, bacterial strains and media

Nicotiana tabacum W38 was obtained through 'Vereinigte Saatzuchten' (Ebstorf, Germany). Plants in tissue culture were grown under a 16 h light/8 h dark regime on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 2% sucrose (2 MS). *Escherichia coli* strain DH5α (Bethesda Research Laboratories, Gaithersburg) was cultivated using standard techniques (Sambrook *et al.*, 1989). *Agrobacterium tumefaciens* strain C58CX1 containing pGV2260 (Deblaere *et al.*, 1985) was cultivated in YEB medium (Vervliet *et al.*, 1975).

Reagents

DNA restriction and modification enzymes were obtained from Boehringer Mannheim (Ingelheim, Germany) and New England Biolabs (Danvers, USA). Chemicals were obtained through Sigma Chemical Co. (St. Louis, USA) or Merck (Darmstadt, Germany).

Recombinant DNA techniques

Standard procedures were used for recombinant DNA work (Sambrook *et al.*, 1989).

Constructs

A diagram of the final constructs is shown in Figure 2. pTetVP16: pTet1 (Gatz *et al.*, 1991), which contains the *tetR* coding region between the CaMV 35S promoter and the octopine synthase (*ocs*) transcriptional terminator, was digested with *Bam*HI and *Sph*I and treated with T4-Polymerase to generate blunt ends. Religation of the vector resulted in the loss of the *Xba*I, *Sal*I, *Pst*I and *Sph*I sites between the coding region and the *ocs* transcriptional terminator. The resulting plasmid pTet1Δ was digested with *Xba*I and *Bam*HI in order to excise *tetR* sequences downstream from amino acid 3. The coding sequence of tTA was gained by *Xba*I/*Bam*HI digestion of pUHD 15-1 (Gossen and Bujard, 1992) and ligated into pTet1Δ cut with *Xba*I and *Bam*HI to yield pTetVP16. pUC-TetVP16: The *tTA* gene under the control of the CaMV 35S promoter and the *ocs* transcriptional terminator was excised from pTetVP16 as an *Eco*RI/*Hin*dIII fragment and inserted into pUC18 cut with *Eco*RI and *Hin*dIII (Figure 1). pUC-Top10: pUC-Top10 was derived from pIGF107 (Gatz *et al.*, 1991), which contains the coding region of the bacterial *chloramphenicol acetyl transferase* (*cat*) gene under the control of a modified CaMV 35S promoter. This promoter contains a number of additional unique restriction sites between positions –1 and –53. The plasmid was linearized with *Spe*I at position –53, the protruding ends were filled in using Klenow DNA polymerase, and a synthetic 55 bp oligonucleotide (Gatz *et al.*, 1991) encoding two operators and a *Bgl*II site was inserted. The promoter fragment was cloned as an *Eco*RI/*Xho*I (fill-in) fragment into pGus (Köster-Töpfer *et al.*, 1989) cut with *Eco*RI and *Sma*I. The resulting plasmid pSpe-Gus was cut with *Asp*718 and *Bgl*II(fill-in) and ligated with the *Asp*718/*Xho*I (fill-in) fragment from pUHC 13-4 (Gossen and Bujard, 1992), so that the CaMV 35S enhancer was replaced by seven *tet* operators. In the course of this step part of the 55 bp oligonucleotide was unintentionally deleted, so that seven *tet* operator sequences (instead of nine) were left. The sequence of this promoter (P-Top10) is shown in Figure 2(b). pUC-Top10-Gus/int: the coding region of the β-glucuronidase (*gus*) gene and the nopaline synthase (*nos*) polyadenylation signal from pUC-Top10 was excised with *Bam*HI and *Hin*dIII and replaced by the coding region of a modified *gus* gene containing an intron (Vancanneyt *et al.*, 1990) and the CaMV 35S polyadenylation signal. pTop10: The *gusInt* gene under the control of the synthetic Top10 promoter was cloned as an *Eco*RI/*Hin*dIII fragment into pBIN19 (Bevan, 1984) cut with *Eco*RI and *Hin*dIII. pTetVP16-Top10: a *Hin*dIII linker was cloned into the *Eco*RI (fill-in) site of pUC-Top10-Gus/int. The *gusInt* gene under the control of the synthetic Top10 promoter was cloned as a *Hin*dIII fragment into pTetVP16 *Hin*dIII. For further experiments we chose a plasmid that contained the *tTA* gene and the *gus* gene tail to tail (Figure 2). pNosTetVP16: pTetVP16 was cut with *Eco*RI and *Asp*718 (fill-in).

The vector fragment was ligated with an *EcoRI/PstI* (made blunt ended with T4-polymerase) fragment encoding the Nos promoter (An *et al.*, 1986). The *gus(int)* gene under the control of the synthetic Top10 promoter was cloned as a *HindIII* fragment into pTetVP16 *HindIII* yielding pNosTetVP16-Top10 (Figure 1).

Binding studies with plant extracts

An operator containing restriction fragment was excised with *EcoRV* and *BglI* from pLUP-1 (Frohberg *et al.*, 1991), end-labeled by filling in the protruding ends in the presence of [α-^{32}P]dATP using Klenow-Polymerase and separated from the vector by polyacrylamide gel electrophoresis and subsequent elution. Binding reaction and gel electrophoresis were done as described (Gatz *et al.*, 1991).

Transient expression in tobacco protoplasts

Isolation and transformation of protoplasts was essentially as described (Gatz *et al.*, 1991)

Northern blot analysis

Total RNA from leaves was prepared according to Logemann *et al.* (1987). Blotting and hybridization was carried out as described (Heyer and Gatz, 1992).

Assays for Gus activity

For the fluorometric Gus assay, explants were homogenized and incubated with the substrate 4-methylumbelliferyl-β-D-glucuronide at 37°C. Quantification of the fluorescence was done according to Jefferson *et al.* (1987). Protein concentrations were determined according to Bradford (1979).

For *in vivo* staining, intact plant material was vacuum infiltrated with 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium) and incubated overnight at 37°C.

Tobacco transformation

Transformation of tobacco plants was carried out using the *Agrobacterium tumefaciens* leaf disc technique as described by Rosahl *et al.* (1987).

Application of Tc to the plants

Plants were regenerated on 1 mg l^{-1} Tc. Vacuum infiltration of single leaves was done as described (Gatz *et al.*, 1991). For Tc uptake through roots, plants were cultivated in a beaker containing Hoagland buffer and 1 mg l^{-1} Tc, which was changed every other day. Oxygene was supplied through an aquarium pump.

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